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EDITORIAL

Hepatic metastases from gastric cancer: A surgical perspective

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Abstract

Management of patients with hepatic metastases as the sole metastatic site at diagnosis of gastric cancer (synchronous setting) or detected during follow-up (metachronous) is controversial. The prevailing attitude in these cases is passive, leading to surgical palliation and, possibly, to chemotherapy. Authors focused this editorial in order to promote a more pragmatic attitude. They stress the importance of recognizing the good candidates to curative surgery of both gastric cancer and hepatic metastases (synchronous setting) or hepatic disease alone (metachronous disease) from those who will not benefit from surgical therapy. In fact, in adequately selected subgroup of patients surgery, especially if integrated in multimodal therapeutic strategies, may achieve unexpected 5-year survival rates, ranging from 10% to 40%. The critical revision of the literature suggests that some simple clinical criteria exist that may be effectively employed in patients selection. These are mainly related to the gastric cancer (factors T, N, G) and to the extent of hepatic involvement (factor H). Upon these criteria it is possible to adequately select about 50% of cases. In the remaining 50% of cases a critical discussion on a case-by-case basis is recommended, considering that among these patients some potential long-survivors exist, that survival is strictly influenced by the ablation of the tumor bulk and by multimodality treatments including chemotherapy and that in expert institutions this kind of surgery is performed with very low mortality and morbidity rates.

Key words: Gastric cancer; Hepatic metastases; Surgical palliation; Therapeutic strategy; Hepatectomy; Selection criteria; Gastrectomy; Chemotherapy

Core tip: Authors highlight the reasons for an active attitude in case of patients with gastric cancer and hepatic metastases. They show that when the liver is the sole metastatic site it is possible to select the good candidates for surgical management of both gastric cancer and hepatic metastases and to recognize those who will not benefit from an aggressive attitude. They also show that the multidisciplinary approach to these patients is the best option.

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Sparkling is research and clinical activity which focuses on hepatic metastases from colorectal cancer, but the waters surrounding hepatic metastases from gastric cancer are still.

In 2005 the French Association of Surgery[1] produced the first report with more than 100 hepatectomies, recruiting 101 cases from 41 centres. In 2010 Kerkar *et al*[2] reviewed 436 patients collected from 19 surgical series published over a 20-year time-span. The most recent review, published by Fitzgerald *et al*[3] collected 481 cases published in the period 1990-2013. Despite this, the incidence of hepatic metastasis from gastric cancer during the course of the disease figures around 20% in eastern countries such as Japan and South Korea and rises to 30%-40% in Europe and North America, lying unmeasured -yet probably over 50%- in other less closely-monitored countries.

It seems that the clinical community does not include surgery among the therapeutic options for these patients. We all have daily experience of the aggressive biology of gastric cancer, especially when at metastatic stage. We also experience the frequent coexistence of multiple metastatic sites as well as hepatic involvement and are also aware that hepatic and systemic recurrence is almost systematically observed after hepatectomy or ablative treatments. However, literature is consistent in reporting unexpected results after aggressive multidisciplinary management including surgery in gastric cancer patients when the liver is the sole metastatic site[4]. This clinical context is observed in about 33% of cases presenting hepatic metastases, but in these cases 5-year survival rate is reported between 10% and 40% of cases.

In other fields of our work, such as pancreatic cancer, surgeons struggle in their theatres for hours, master difficult techniques, face perilous postoperative complications and accept surgical mortality for similar but often worse results. Why is this not the case for gastric cancer with hepatic metastases as the only metastatic site?

The main reason may be found in the curve that describes survival after surgery: a step drop is systematically observed during the first year, mortality is around 40% after 6 mo and reaches 70%-80% 1 year after surgery. This suggests the critical point: selection of candidates to curative surgery. All papers published on this topic investigate selection criteria and prognostic factors of major clinical relevance. However, thirteen of them[5-16] are of great interest since their results are based on cohorts of patients as observed in every-day clinical practice and not upon super-selected populations submitted to surgical treatment.

In the different settings of the disease, synchronous and metachronous presentation, simple clinical variables such as factor T of gastric primary and extension H of hepatic involvement may be employed to select the best candidates for curative surgery[6,7,10,15,16] and those to be excluded from hepatic resection. These factors also seem to display a cumulative effect. In the synchronous setting[6] gastric cancer T > 2 and scattered bilobar metastases (H3) are negative prognostic factors with clear clinical value. In fact, median and 5-year survival was respectively 23 mo and 27% for the 10% of cases which did not display the 2 risk factors, while patients affected by T $\geqslant$ 3 gastric cancer and H3 metastases (30% of cases) displayed a median survival of 6 mo and did not survive more than 16 mo. Accordingly, in the metachronous setting[10] the variable T4, N+ and G3 showed a negative prognostic role. Patients not presenting these variables (7%) had a 5-year survival rate of 40%, those affected by two or three negative prognostic factors (48%) had a median survival of 4 ± 3 mo. Upon these bases, it is possible to select the best candidates for curative resection, those for whom an aggressive treatment should be mandatory, from those who will not benefit from hepatectomy. All together, they represent 40%-55% of cases. In the middle one finds the huge group of cases presenting 1 risk factor. They do not display an astonishing survival performance (median survival is around 8-9 mo). Yet among these it is possible to find long-term survivors. We think that in these cases the therapeutic decision should be discussed on a case by case basis, considering that the major prognostic factor emerging from the cited papers is represented by the possibility to achieve a curative resection. This should be pursued whenever possible, also referring to ablative procedures such as RFA[5,8]. It must be noted that in tertiary Institutions these complex procedures are safe: in both the synchronous and metachronous setting mortality is limited to some unit percent (0%-3%) and morbidity reflects that of major surgery, well under 20% of cases.

We would like to stress here that the completeness of tumor bulk removal is a key-point of the therapeutic strategy. The expansion of the experience and the most recent series focusing on surgical subgroups[1,17-25], indicate this point precisely. The importance of a radical resection/ablation of gastric cancer and hepatic metastases stands out progressively while other prognostic factors reveal themselves as disturbing and non-existent[4].

A pragmatic multi-disciplinary approach, integrating neo-adjuvant and/or adjuvant chemotherapy, offers the possibility for further improvements in results. In our recent paper[10], adjuvant chemotherapy revealed itself as the most powerful prognostic factor. The effective integration of the different disciplines will be the next breakthrough although it will require a great deal of hard work. Though a paradox, chemotherapy with neo-adjuvant intent is not routinely accepted in metastatic settings, especially in synchronous presentation, and patients are more often enrolled in protocols of palliative chemotherapy.

Concluding this editorial, we repeat that through slow but constant progress it is possible today to operate a certain selection of candidates to curative resection and that complete removal of the neoplastic bulk must be achieved. It is relatively easy to recognize the best candidates to be operated on or - at least - to be centralized in expert centres, where these complex procedures can be performed with very low operative morbidity and mortality. Upon this basis, we hope that a μετάνοια (change of mind) will spread through our community, leading to a general and consistent improvement of survival results, at least for some of the most unlucky among gastric cancer patients.

Will we accept the challenge?

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Immunomodulating effects of antibiotics used in the prophylaxis of bacterial infections in advanced cirrhosis

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Abstract

The use of norfloxacin either as primary or secondary prophylaxis of bacterial infections in advanced cirrhosis has improved patient's survival. This may be explained not only due to a significant decrease in the number of infections, but also because of a direct immunomodulatory effect. Selective intestinal decontamination with norfloxacin reduces translocation of either viable bacteria or bacteria-driven products from the intestinal lumen. In addition, norfloxacin directly modulates the systemic inflammatory response. The pro-inflammatory cytokine profile secreted by neutrophils from these patients shows a close, significant, and inverse correlation with serum norfloxacin levels. Similar effects have been described with other quinolones in different clinical conditions. Although the underlying mechanisms are not well defined for most of the antibiotics, the pathways triggered for norfloxacin to induce such immunomodulatory effects involve the down-regulation of pro-inflammatory inducible nitric oxide synthase, cyclooxygenase-2, and NF-κB and the up-regulation of heme-oxygenase 1 and IL-10 expression. The knowledge of these immunomodulatory effects, additional to their bactericidal role, improves our comprehension of the interaction between antibiotics and the cellular host response and offer new possibilities for the development of new therapeutic strategies to manage and prevent bacterial infections in cirrhosis.

Key words: Cirrhosis; Prophylaxis; Cytokines; Bacterial DNA; Norfloxacin

Core tip: The use of antibiotic therapy to either treat or prevent frequent bacteria-derived complications arising in patients with cirrhosis is well established. The

knowledge of antibiotic immunomodulatory mechanism, additional to their bactericidal role, improves our comprehension of the interaction between these molecules and the cellular machinery, and provides insight on the development of alternative strategies in the management and prevention of bacterial infections in cirrhosis.

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BACTERIAL INFECTIONS IN CIRRHOSIS

Bacterial infections are among the most frequent complications arising in patients with cirrhosis and are associated with clinical consequences such as failure to control bleeding from oesophageal varices[1] and reduced survival[2]. Up to 35% of patients with cirrhosis develop nosocomial infections whereas this percentage is significantly lower (5%) in the general population[3].

Bacterial infection episodes are mainly caused by Gram-negative bacilli of enteric origin. Bacterial species such as *Escherichia coli* (*E. coli*) are responsible for spontaneous bacterial peritonitis (SBP) episodes, urinary tract infections or pneumonia in cirrhosis. The accepted pathogenic mechanism to explain the passage of bacteria or their products from the intestinal lumen through the intestinal wall and to mesenteric lymph nodes (MLNs) is defined as bacterial translocation (BT)[4,5]. Spontaneous BT, evaluated either by culture-positive MLNs or the presence of surrogate markers in blood such as lipopolysaccharide (LPS)-binding protein (LBP) or bacterial DNA, has been reported to occur in 30%-35% of patients with cirrhosis[6-8].

Intestinal bacterial overgrowth (IBO), increased intestinal permeability and immune alterations are considered as the main mechanisms involved in the pathogenesis of BT in cirrhosis (Figure 1). Experimental studies have shown a higher rate of IBO in cirrhotic rats with ascites and BT compared with those without BT[9,10]. However, these studies also report a significant percentage of animals showing IBO without BT, suggesting the implication of other factors in the pathogenesis of BT.

The gut barrier integrity is also considered as an important issue in preventing BT. Recent studies have shown the relevance of maintaining gut barrier integrity as a potential target to modify/reduce BT episodes in experimental cirrhosis. Increased intestinal permeability has been associated with increased serum endotoxin levels in different experimental models of liver damage[11] and the gut barrier protective role of certain probiotics have succeed in decreasing induced BT in CCl4-cirrhotic mice[12] and rats[13]. However, therapy against IBO without interfering intestinal permeability has been shown to decrease the rate of BT in cirrhotic rats[14], suggesting that the increase of intestinal permeability may not be sufficient for explaining BT pathogenesis

Finally, translocating bacterial products into MLNs or to portal blood are usually phagocyted and neutralized, but alterations in local and systemic immunity may favour the development of BT episodes. For example, SBP has been correlated with nucleotide-binding oligomerization domain containing protein 2 (NOD2) gene variants that fail in the recognition of bacterial peptidoglycans therefore affecting local immunity[15].

BACTERIAL TRANSLOCATION AND INFLAMMATION

Decompensated cirrhosis is associated with impaired cellular and humoral immune responses[16,17]. For instance, decreased serum levels of Complement subunit C3 predispose to develop SBP[18]. Cirrhosis and the development of SBP has also been correlated with a decreased phagocytic activity of the reticuloendothelial system[19]. In addition, the translocation of bacteria or their products induces a chronic inflammatory response that may aggravate the haemodynamic disturbances observed in cirrhosis[20].

Our group has extensively worked on the systemic inflammatory mechanisms and consequences of the passage of bacterial products into the blood of patients with decompensated cirrhosis. The cell mediated immune response and nitric oxide production is activated in peritoneal macrophages from patients with cirrhosis and ascites who show translocation of bacterial DNA into blood and ascitic fluid (AF)[21], and the relevance of the inflammatory response is species-specific in serum and AF of these patients[22]. Also, the translocation of bacterial DNA induces the activation of the complement system in patients with advanced cirrhosis[23], probably justifying its consumption when repeated episodes occur[24]. We have reported as well an ERK-related signalling pathway activated in peritoneal macrophages in response to bacterial DNA[25], the similarity between the inflammatory reaction induced by bacterial DNA translocation and that observed in overt infections such as SBP[26] and the detection of bacterial-synthesized peptides in the ascitic fluid of patients with decompensated cirrhosis associated with an increased pro-inflammatory cytokine cascade[27]. In addition, other bacterial products such as LPS have widely shown their immunogenic effect on patients with cirrhosis[28]. All this evidence clearly state not only the translocation of viable bacteria but also the translocation of bacterial products as an

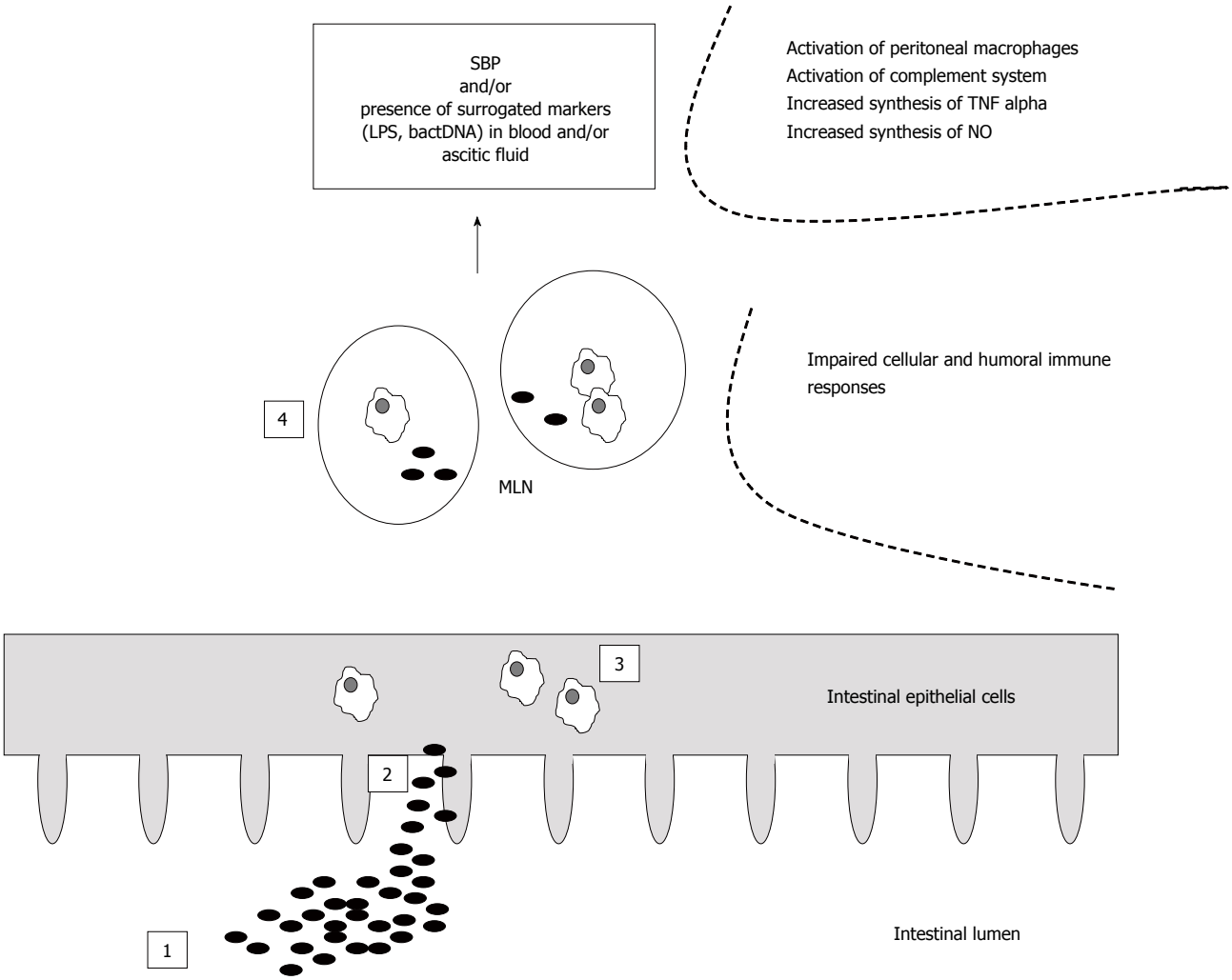

Figure 1 Bacterial translocation and spontaneous bacterial peritonitis - pathogenic mechanisms. Intestinal bacterial overgrowth (1), increased intestinal permeability (2) and immune alterations (3 and 4) are considered as the main mechanisms involved in the pathogenesis of bacterial translocation (BT) defined as the passage of bacteria from the intestinal lumen through the intestinal wall and to mesenteric lymph nodes (MLNs) in cirrhosis. SBP: Spontaneous bacterial peritonitis.

immunogenic mechanism directly implicated in the engagement of systemic inflammation in cirrhosis.

Other studies focused on mucosal inflammation have demonstrated that the synthesis of TNF-α and nitric oxide exacerbates oxidative damage in the intestinal mucosa[29], which in turn increases intestinal permeability probably favouring BT and creating a feedback that perpetuates inflammation. In fact, our group has demonstrated that the administration of anti-TNF-α monoclonal antibodies to cirrhotic rats with ascites is associated with a significant decrease in the rate of BT[30].

SELECTIVE INTESTINAL DECONTAMINATION

Selective intestinal decontamination (SID) consists of the aerobic Gram-negative bacteria clearance from intestinal content with oral non-absorbable or poorly absorbable antibiotics. Norfloxacin is the most frequently used antibiotic for long-term SID. Norfloxacin has been proved as poorly absorbable and specifically active against Gram-negative bacteria. In addition, the incidence of adverse effects associated with its long-term administration is low[31].

Norfloxacin is a synthetic 6-fluoro 4-quinolone molecule that antagonizes enzymatic activity of DNA gyrase of bacteria[32]. Norfloxacin is well tolerated according to randomized controlled trials performed[33], it is excreted through the kidneys and the usual pharmacological parameters, such as serum half-life or peak concentration, are not altered in patients with moderate liver damage compared with healthy controls[34].

In the setting of cirrhosis, oral norfloxacin is administered, either as primary prophylaxis (400 mg twice a day for 1 wk) to patients with variceal bleeding, severely decompensated cirrhosis and those with ascites protein concentration of < 15 mg/L or as secondary prophylaxis (indefinitely, 400 mg daily) to those who have survived a previous episode of SBP[35]. In this last condition, norfloxacin administration significantly reduces the incidence

of bacterial infections[31,36] and, used as primary prophylaxis, also reduces noninfectious related clinical complications, such as hepatorenal syndrome, thus improving survival[35,37]. Oral ciprofloxacin 500 mg/d is an alternative option to norfloxacin[38]. On the other hand, although norfloxacin decreases certain virulence factors even in quinolone-resitant *E. coli*, long-term norfloxacin prophylaxis increases 2.7 fold the risk of developing multiresistant bacterial infections and almost 4 fold the risk of infections caused by extended-spectrum β -lactamase-producing *Enterobacteriacea*[39,40].

Recently, rifaximin, an antibiotic with broad-spectrum antimicrobial activity that eliminates intestinal flora non-selectively has been suggested as an alternative in the prophylaxis of bacterial infections in cirrhosis. This antibiotic reaches high fecal concentrations but is virtually non-absorbed (bioavailability in blood after oral administration < 0.4%), reduces the expression of bacterial virulence factors and compromises plasmid transfer and, despite high gut concentrations and its broad spectrum of activity, produces minimal alterations in the intestinal microflora[35]. Nevertheless, there are no studies comparing rifaximin *vs* norfloxacin in the prevention of SBP.

Efficacy of norfloxacin in preventing recurrent SBP therefore relies on the removal of enteric Gram-negative bacteria, which are mainly responsable for SBP episodes occuring in patients with cirrhosis (Figure 2). Although norfloxacin is poorly absorbable, its long-term administration upholds serum levels higher than 90% of minimal inhibitory concentration for most aerobic Gram-negative[41].

ANTIBIOTICS AND INFLAMMATORY DOWNREGULATION

Most antibiotic classes exert effects on the immune system and complement activation. Sulphonamides, tetracyclines, beta-lactams, aminoglycosides, lincosamides, chloramphenicol, rifampin, benzylpyrimidinones, fusidic acid, fosfomycin or gyrase B-inhibitors, and, in particular, macrolides and fluoroquinolones either increase or decrease phagocyte functions. For most of this antibiotics the underlying mechanisms of immunomodulation are not well defined[42].

Fluorquinolones are able to concentrate intracellularly in human phagocytic cells on average 3 to 12-fold depending on the particular cell type and fluoroquinolone studied[43]. This intracellular accumulation of fluoroquinolones is modulated in macrophages by the activity of drug efflux pumps. Thus, gemfibrozil and probenecid, inhibitors of multidrug resistance proteins (MRP) and other transporters of organic anions, increase the intracellular accumulation of ciprofloxacin[44]. Numerous studies have shown that intracellular fluorquinolones remain active against facultative and obligate intracellular pathogens such as mycobacteria, *Legionella* spp, *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Staphylococcus aureus* despite the acidic intracellular pH. However, little is known about how and to what extent this intracellular accumulation of antibiotics directly and specifically relates to changes in phagocytic and immunomodulatory activity of macrophages[45].

Ciprofloxacin improved survival in mice exposed to sublethal LPS challenge changing the pattern of early cytokine response. In the absence of ciprofloxacin LPS administration resulted in peaks of serum TNF-α and IL-10 concentrations at 1 h that disappeared by 6 h while serum IL12 concentrations peaked at 3 h and decreased by 6 h. Pre-treatment with a dose of ciprofloxacin prevented death in these mice, and consistently and significantly attenuated the TNF-α burst, decreased IL-12 concentrations and increased the IL-10 response. Similarly, trovafloxacin reduced TNF-α levels and decreased mortality in a experimental model of intra-abdominal abscesses in rats despite subtherapeutic doses of the drug were used or animals were challenged with heat-killed bacteria[46]. These results demonstrate that fluoroquinolones affect cytokine responses to bacterial products, an effect that may be particularly important when considering the consequences of early inflammatory responses to infection when cytokines influence the functional differentiation of T lymphocytes to initiate the acquired immune response[47].

Several clinical and experimental studies indicate that the fluoroquinolones exert immunomodulatory activities in latent or chronic infections affecting the synthesis of cytokines. Chronic infections such as those caused by *Chlamydia pneumoniae* are characterised by a marked inflammatory response, mediated by NF-κB and other transcription factors. Fluoroquinolones show a direct antichlamydial activity and simultaneously produce a reduction in secretion of proinflammatory cytokines *via* NF-κB necessary to achieve efficacy in this setting[45]. On the other hand, the participation of activated macrophages in the resolution of *Listeria monocytogenes* infections is determined by interferon, which acts synergistically with sparfloxacin and moxifloxacin[48]. *Staphylococcus aureus* excretes a wide array of toxins including superantigens, which cause T-cell proliferation, the release of cytokines, and promote apoptosis increasing TNF-RI and Fas receptor expression and Fas ligand production. Moxifloxacin inhibits apoptosis and downregulates the staphylococcal superantigen induced mRNA expression of Fas, FasL, and TNF-RI[45]. In general, these studies indicate that the fluoroquinolones exert immunomodulatory activities besides their own bactericidal properties supporting further their efficacy in latent or chronic infections.

In cirrhotic patients, SID with norfloxacin reduces translocation of either viable bacteria or bacteria-

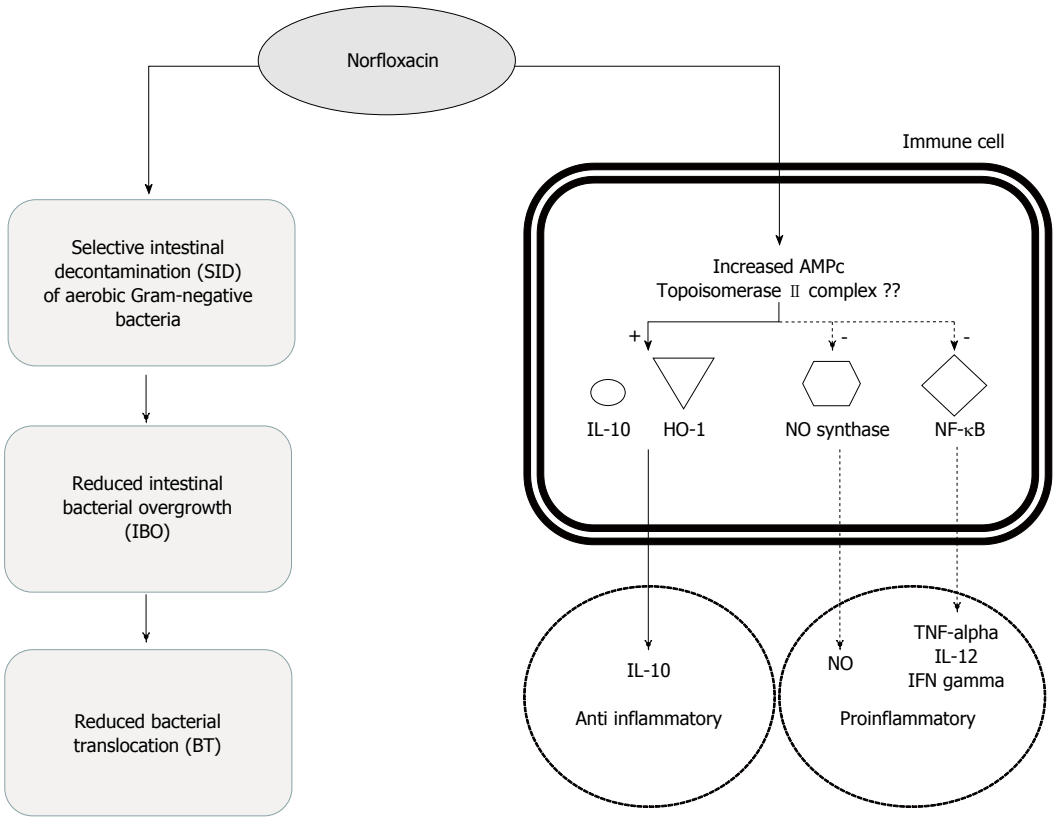

Figure 2 Immunomodulatory and bactericidal effects of norfloxacin in patients with cirrhosis.

driven products from the intestinal lumen and then modulates the immune system activity but also could modulates patients' proinflammatory reaction by acting directly on phagocyte response as described in other settings (Figure 2). In fact, we have shown that norfloxacin but not trimethoprim/sulfamethoxazole modulates inflammatory response and directly affects neutrophils activity in patients with cirrhosis. Indeed, serum levels of proinflammatory cytokines TNF-α and IL12 and interferon-γ showed a close, significant, and inverse correlation to serum norfloxacin levels, both at the peak time and at trough stages of the drug concentrations[41]. During the first 4 h after drug administration, when maximum serum and intracellular norfloxacin levels are reached, the transcription factor responsible of transcription of proinflammatory genes and cytokine synthesis as TNF-α and interleukin 8 (NF-κB) is down-regulated. These evidences are in agreement with the low cytokine levels frequently observed in patients with cirrhosis and ascites undergoing SID with norfloxacin[41]. Neutrophils from patients with cirrhosis cultured with LPS showed an up-regulation in IL-10 levels and heme oxygenase-1 expression in patients receiving norfloxacin. IL-10 levels, HO-1 expression and norfloxacin concentrations were directly correlated, whereas proinflammatory inducible nitric oxide synthase, cyclooxygenase-2, and NF-κB were inversely correlated[49]. All these data are consistent with the effects of other quinolones in different clinical conditions and experimental models supporting the hypothesis of the existence of an immunomodulatory effect of norfloxacin in cirrhotic patients that acts synergistically with direct antibiotic effect on the intestinal bacterial flora.

Liver transplantation represents a particular situation characterized by immunosuppression and a high risk of infections associated to an increase in mortality, morbidity, and hospital stay. As a consequence, many patients are subjected to antibiotic prophylaxis. No significant differences in the number of infections between intervention and control groups were found in all seven low-quality trials published up to now[50]. The effect of antibiotics on post-transplantation immunosuppression status is unknown.

It has been proposed that increased levels of cytokines, such as TNF-α, IL12, IL-1 and IL-6, and IFN-γ play a role in the hyperdynamic circulatory syndrome of cirrhosis. In mice the hypotension elicited by TNF-α is reversed after inhibition of NO synthesis, suggesting that l-arginine-derived NO is a principal mediator of TNF-α-induced hypotension[51]. It has been described in experimental cirrhosis that TNF-α induces NO synthesis increasing the activity of the inducible and endothelial forms of NO synthase (iNOS and eNOS, respectively). Several studies in patients with cirrhosis have showed a positive effect of norfloxacin on vascular resistance and pulmonary shunting, reversing the hyperdynamic circulation, increasing mean arterial blood pressure and systemic vascular

resistance[52]. In patients with cirrhosis treated with norfloxacin for SID and in parallel to proinflammatory cytokines concentrations, serum NO shows a close, significant, and inverse correlation to serum norfloxacin levels suggesting an effect of norfloxacin regulating cytokine response and then NO production and associated haemodynamic changes[41].

MECHANISM/S

Norfloxacin is a synthetic 6-fluoro-4-quinolone which primary bacterial target is bacterial DNA gyrase or topoisomerase Ⅱ. Bacterias require DNA gyrase for DNA replication, transcription of certain genes and aspects of DNA repair and recombination. All these activities are antagonized by fluoroquinolones[53]. However, the basic mechanisms underlying their immunomodulatory activity have not been elucidated.

It has been postulated that TNF-α inhibition could be related to changes in intracellular levels of cyclic AMP (cAMP) (Figure 2). In human monocytes with or without the addition of lipopolysaccharide the presence of ciprofloxacin was associated to a significant accumulation of cAMP[54]. Similarly, 2-phenyl-4-quinolone (YT-1), a quinolone compound that did not activate adenylate cyclase but inhibited the cytosolic phosphodiesterase activity, increased the cellular cAMP levels and the cAMP-protein kinase A (PKA) activity in rat neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP)[55]. These studies suggest evidence that fluoroquinolones can directly inhibits phosphodiesterase leading to accumulation of cAMP and enhanced PKA activity. Activation of PKA is associated with intracellular protein phosphorylations and transcription factors activation, and suppression of TNF-α expression. Another direct effect on cytosolic systems of fluoroquinolones has been hypothesized after findings suggesting that ciprofloxacin induced the production of PGE2 in monocytes in a concentration-dependent manner enhancing the expression of cyclooxygenase (COX-2) protein and the elevation of intracellular cAMP in monocytes[56] and after data showing a parallel increase in IL-10 levels and heme oxygenase-1 expression in patients receiving norfloxacin[49], suggesting the involvement of the pathway 1L-10/heme oxygenase 1, a pathway profoundly effective in dampening the enhanced activation of innate immune responses, in the fluoroquinolone effect (Figure 2).

Most experimental models studying the relationship between cytokine expression and quinolone exposition show that alteration in cytokine production correlated with parallel changes in the concentration of mRNA of the specific cytokine. These mRNA alterations were seen only when cells were stimulated by stimuli such as phytohaemagglutinin, lipopolysaccharide or phorbol-myristate acetate (PMA) before quinolone exposure[47].

NF-κB is the crucial factor in mRNA transcription of many cytokines and chemokines including TNF-α and interleukin 8 molecules. It also has an important role in other central cellular events such as superoxide production and apoptosis. In resting cells, NF-κB is bound to an inhibitory protein, inhibitory kappa B (IκB), and retained in the cytoplasm. Cell stimulation with several agonists triggers signal transduction pathways that ultimately result in activation of IκB kinases (IKK). Phosphorylation of IκB by IKK is followed by a rapid degradation of the IκB proteins thereby freeing NF-κB, which then enters the nucleus, binds to DNA, and activates transcription[45].

In a model of reactivation of latent HIV-1 expression in chronically infected promonocytic U1 cells, ciprofloxacin (at concentrations between 1.5 and 10 mg/L) inhibited HIV-1 expression in PMA-stimulated U1 cells. This effect was associated with a significant decrease in TNF-α production and with significant inhibition of NF-κB[57]. Moxifloxacin-treated mice showed no bronchopneumonia after cyclophosphamide injection and intra-tracheal inoculation of C albicans. This protective effect was associated with a significant inhibition of TNF-α and interleukin 8 secretion compared with controls. In lung tissue analysed by immunohistochemistry, moxifloxacin-treated animals showed a marked and significant decrease of NF-κB staining in epithelial cells and macrophages. When human monocyte cell lines were stimulated by lipopolysaccharide a significant increase in interleukin 8 and a significant degradation of IκB were observed. Both effects were abolished when cells were exposed to moxifloxacin[58]. These results indicate that moxifloxacin inhibits NF-κB activation, probably through its inhibitory effects on IκB degradation. According with these studies we have observed in cirrhotic patients treated with norfloxacin a NF-κB down-regulation in parallel to increases in quinolone levels and to decreases in TNF-α concentrations (Figure 2). Then norfloxacin shows in this setting a similar immunomodulatory effect to that described for other fluoroquinolones[41].

In eukaryotic cells quinolones target the topoisomerase Ⅱ complex in a similar manner to prokaryotic cells but their inhibitory effects is generally attained at concentrations that are two to three orders of magnitude higher[59]. As in prokaryotic cells, quinolones were shown to result in an increased formation of topoisomerase Ⅱ-cleaved DNA complexes. If intranuclear processes relating to quinolone-topoisomerase Ⅱ interaction may have an effect on cytosolic activation or inhibition of NF-κB requires further research (Figure 2).

FUTURE DIRECTIONS

The use of norfloxacin either as primary or secondary prophylaxis of bacterial infections in advanced cirrhosis has improved patient's survival, not only due to a

significant decrease in the number of infections, but also in the incidence of other deadly complications such as hepatorenal syndrome. Quinolones not only exert their beneficial effect through bactericidal mechanisms, as shown above, but also by an immunomodulatory effect. The extent to which this immunomodulatory effect actually contributes to norfloxacin efficacy in cirrhotic patients requires further research.

On the other hand, long-term norfloxacin prophylaxis has been related with an increased risk of developing multiresistant bacterial infections being this effect the main factor that can limit the use of this prophylactic strategy in the near future. New studies are needed to assess the possible involvement of the changes on the immune system exerted by fluoroquinolones in the development of these resistances. Related to this, it is necessary to study whether different strategies to modulate the activity of the immune system as well as new drugs with different effects not only on bacteria but also on immune cells can prevent and/or treat the development of multiresistant nosocomial bacterial infections in cirrhotic patients. In fact this situation is considered so relevant that recently a consortium comprised by more than 30 European companies and universities have joined efforts to develop new antibiotics against gram-negative bacteria, a European funding project from the Innovative Medicines Initiative.

Until new drugs are available however, the intimate knowledge of immunomodulatory activities exerted by antibiotics at intracellular levels may improve survival in patients with cirrhosis and open interesting possibilities for future researches.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Cirrhotic cardiomyopathy

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Abstract

During the course of cirrhosis, there is a progressive deterioration of cardiac function manifested by the disappearance of the hyperdynamic circulation due to a failure in heart function with decreased cardiac output. This is due to a deterioration in inotropic and chronotropic function which takes place in parallel with a diastolic dysfunction and cardiac hypertrophy in the absence of other known cardiac disease. Other findings of this specific cardiomyopathy include impaired contractile responsiveness to stress stimuli and electrophysiological abnormalities with prolonged QT interval. The pathogenic mechanisms of cirrhotic cardiomyopathy include impairment of the b-adrenergic receptor signalling, abnormal cardiomyocyte membrane lipid composition and biophysical properties, ion channel defects and overactivity of humoral cardiodepressant factors. Cirrhotic cardiomyopathy may be difficult to determine due to the lack of a specific diagnosis test. However, an echocardiogram allows the detection of the diastolic dysfunction and the E/e' ratio may be used in the follow-up progression of the illness. Cirrhotic cardiomyopathy plays an important role in the pathogenesis of the impairment of effective arterial blood volume and correlates with the degree of liver failure. A clinical consequence of cardiac dysfunction is an inadequate cardiac response in the setting of vascular stress that may result in renal hypoperfusion leading to renal failure. The prognosis is difficult to establish but the severity of diastolic dysfunction may be a marker of mortality risk. Treatment is non-specific and liver transplantation may normalize the cardiac function.

Key words: Cirrhotic cardiomyopathy; Inotropic heart dysfunction; Left ventricular diastolic dysfunction; E/e' ratio; Arterial blood volume; Cirrhosis; Liver failure; Hepatorenal syndrome

Core tip: During the course of cirrhosis, there is an impairment in cardiac function with decrease in cardiac output. This process is due to a cirrhotic cardiomyopathy with diastolic dysfunction that may compromise the inotropic function which takes place

in parallel with a chronotropic heart dysfunction. This cardiomyopathy plays an important role in the pathogenesis of the impairment of effective arterial blood volume in cirrhosis. The clinical consequences of cardiac dysfunction may be an inadequate cardiac output in response to clinical events that produce effective hypovolemia leading to renal failure. The severity of cardiomyopathy is a marker of advanced cirrhosis and mortality.

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INTRODUCTION

Patients with cirrhosis develop a progressive impairment in their circulatory and cardiac function during the course of their illness. The existence of a systemic circulatory disorder in liver cirrhosis was described more than 60 years ago by Kowalski *et al*[1] and Murray *et al*[2]. Both authors defined a hyperdynamic state in patients with cirrhosis characterized by high cardiac output (CO), plasma volume as well as a decreased systemic vascular resistance (SVR) and blood pressure. In addition to systemic circulatory dysfunction, the clinical course of patients with liver disease is complicated by a progressive impairment in heart function. Cardiac function abnormalities in cirrhosis are clinically not apparent, probably because of the low SVR presented by these patients, which reduces the cardiac afterload. Initially the impaired left ventricular (LV) performance in cirrhotic patients was thought to be due to the direct toxic effect of alcohol[3]. However, data from investigations performed since 1980s show that the blunted cardiac responses to diverse stimuli is not the result of alcohol. These findings support the concept of a specific heart disease termed "cirrhotic cardiomyopathy" (CCM)[4]. Therefore, CCM is an entity clinically and pathophysiologically different from an alcoholic cardiomyopathy. Our review provides an overview of CCM, its definition, possible pathogenic mechanisms, clinical relevance and management.

DEFINITION OF CIRRHOTIC CARDIOMYOPATHY

According to the 2005 World Congress of Gastroenterology, CCM is a chronic cardiac dysfunction characterized by impaired contractile responsiveness to stress stimuli[5,6], and/or impaired diastolic relaxation[7,8], and electrophysiological abnormalities with prolonged QT interval[9], in the absence of other known cardiac disease. On the other hand, patients with cirrhosis display primarily left ventricular diastolic dysfunction (LVDD) with normal systolic function at rest.

FACTORS RELATED TO THE INDUCTION OF CIRRHOTIC CARDIOMYOPATHY

Heart wall thickness changes are common in patients with cirrhosis and portal hypertension. These abnormalities may be an adaptive response to the hyperdynamic circulation and the trophic effects of several neurohumoral systems. In addition, the clinical evidence indicates a link between the degree of liver insufficiency and the severity of CCM. A recent study in advanced cirrhosis documented an association between the extent of CCM and the Model for End-Stage Liver Disease (MELD) score[10]. Changes in diastolic function appear most prominent in patients with severe decompensation. Patients with ascites have worse LVDD compared to those without ascites. Further, the authors showed lack of response of the LV systolic and chronotropic function to peripheral arterial vasodilation and activation of the sympathetic nervous system (SNS)[10]. Therefore, hepatocellular failure and portal hypertension have been considered as possible factors for cardiac changes in patients with cirrhosis.

Cardiac dysfunction in patients with cirrhosis occurs in the setting of a circulatory dysfunction characterized by a marked splanchnic arterial vasodilation. At the initial stages of cirrhosis, the circulatory dysfunction is compensated by the development of a hyperdynamic circulation. Later, during the course of the disease, the progression of liver disease and portal hypertension results in progressive vasodilatation, leading to reduction in the effective arterial blood volume which, in turn, activates the renin-angiotensin-aldosterone system (RAAS) and the SNS[11]. These circulatory changes can lead to the cardiac dilatation of the left chambers[12] and the development of functional changes in the heart. High norepinephrine levels are known to cause impairment of β-adrenergic receptor function.

Factors other than the SNS activity and aldosterone have been implicated in the pathogenesis of cardiac dysfunction in cirrhosis, including nitric oxide (NO), carbon monoxide (CMO) and endogenous cannabinoids[13]. Accumulation of these substances through porto-systemic shunts could act as negative inotropic agents and also participate in the pathogenesis of LVDD in CCM[14].

Inflammation may play an important role in the pathogenesis of cardiac dysfunction specially in decompensated cirrhosis. It has been postulated that intestinal bacterial overgrowth, altered gut permeability and bacterial translocation (*i.e.*, lipopolysaccharide, bacterial DNA) from the intestinal lumen to the circulation may exert continuous pressure on the immune system[15,16]. Specialized receptors

of monocytes and lymphocytes recognise those factors and release inflammatory mediators such as cytokines, reactive oxygen and nitrogen species[17]. These humoral factors may exert inhibitory effects on LV function. Cytokines can affect myocardial function *via* the effects on both the myocyte contractility and the extracellular matrix[18]. In addition to their effect on myocardial remodeling, cytokines have been shown to have direct and indirect effects on myocardial function.

Other factors like alterations in lipid metabolism may also participate in the pathogenesis of CCM. Lipid metabolic abnormalities in patients with cirrhosis facilitate the incorporation of cholesterol into cell plasma membranes. The major factors which lead to the elevated membrane cholesterol content in cirrhosis are probably an increase in plasma cholesterol levels and a decrease in blood lecithin cholesterol acyltransferase activity[19,20]. Alterations in membrane physical properties play an important role in the impaired β-adrenoceptor[21] and ion channel function[22] in the hearts of cirrhotic rats.

PATHOGENIC MECHANISMS

Cardiovascular autonomic dysfunction

Cardiac contractility is regulated primarily by the SNS through β-adrenergic receptors (βAR). Cardiovascular autonomic dysfunction is frequent in advanced cirrhosis[23]. The incidence of autonomic neuropathy varies from 35% to 80%[24,25] and is related to the severity of hepatic dysfunction[26,27]. Autonomic and cardiac dysfunction includes impaired baroreflex sensitivity and heart rate variability[28-30]. Impaired cardiac response to standing is the most frequently abnormal test and is probably due to blunted baroreflex function[31] in the setting of increased activity of the SNS. The major triggers of the SNS overactivity appear to be baroreceptor-mediated stimulation owing to reduced central and arterial blood volumes[32]. Enhanced sympathetic tone with increased cellular exposure to noradrenalin for longer periods may cause myocardial injury, receptor internalization, sequestration, and down regulation which results in a decrease of β-adrenergic receptor density on the plasma membrane[21].

β-adrenergic receptor function

The βAR system is critical in modulating the contractility of cardiac muscle cells. Activation of βAR by epinephrine and norepinephrine couples with Gs-protein and leads to the stimulation a membrane-bound adenylate cyclase and the subsequent release of cAMP. The Gs protein also promotes the direct activation of the calcium channel of the sarcolemma. The second messenger, cAMP, activates a cAMP-dependent protein kinase A (PKA). PKA phosphorylates several intracellular proteins such as L-type calcium channels, phospholamban, troponin Ⅰ, ryanodine receptors thus leading to Ca^{2+} entering the cell. The cytosolic-free Ca^{2+} binds to the protein troponin C and interacts with tropomyosin between the actin and myosin filaments with allows the contraction of the myofibrils (systole) (Figure 1). Readers interested in detailed descriptions of cellular mechanisms are referred to recent reviews[33-36].

Models in experimental studies with rats have shown several abnormalities in the cardiomyocyte β-adrenergic signalling pathway all of which negatively affect contractility. Several studies have shown decreased βARs density and receptor desensitisation as well as impaired cAMP G-protein and adenylyl cyclase production in cardiocytes of cirrhotic rats[37]. Gerbes *et al*[38] found that lymphocytes of decompensated cirrhotic patients also present decreased abundance of βARs which correlates with the cardiac contractility.

Membrane alteration

Changes in membrane fluidity have also been observed in cirrhotic patients as well as in experimental cirrhosis. It has been demonstrated that the fluidity of plasma membranes from the erythrocytes in cirrhotic patients becomes more rigid and less permeable with increases in membrane cholesterol content[39]. These metabolic abnormalities in the plasma membrane of cardiac myocyte interfere with the activation of βAR and calcium channels embedded in the membrane[40]. Decreased plasma membrane fluidity and an abnormal gene expression of the β-adrenergic system at postreceptor level[41] in a bile duct-ligated (BDL)-cirrhotic rat model is associated with reduced Gs-protein levels which implies attenuation of adenylate cyclase and subsequent cAMP production after administration of isoproterenol[21]. Changes in membrane cholesterol content may alter other activities of cardiac sarcolemmal enzymes Na^+-K^+ ATPase, Mg^{2+} ATPase, Ca^{2+} pump ATPase and Ca^{2+}-dependent K^+ channels and Na^+/Ca^{2+} exchanger[42]. Moreau *et al*[43] have shown altered control of vascular tone by Ca^{2+} and K^+ channels. Such alterations in membrane properties are likely to play an important role in inducing ECG abnormalities in cirrhosis. The altered membrane fluidity may also impair stimulation of cardiac muscarinic acetylcholine receptors (M-ChR) that modulate pacemaker activity *via* I_f and $I_{K.ACh}$, atrioventricular conduction, and directly or indirectly force of contraction. Alterations of cardiac M_2-ChR responsiveness and defective signal transduction to cAMP has been reported in experimental cirrhosis[44]. Finally, cardiac dysfunction may be due to alteration in the Ca^{2+} handling in the myocyte. A decrease in L-type calcium channels has been reported in BDL-rat model myocytes whereas the intracellular calcium system appears intact[45].

Humoral cardiodepressant factors

Mikulic *et al*[46] have shown that plasma from cirrhotic

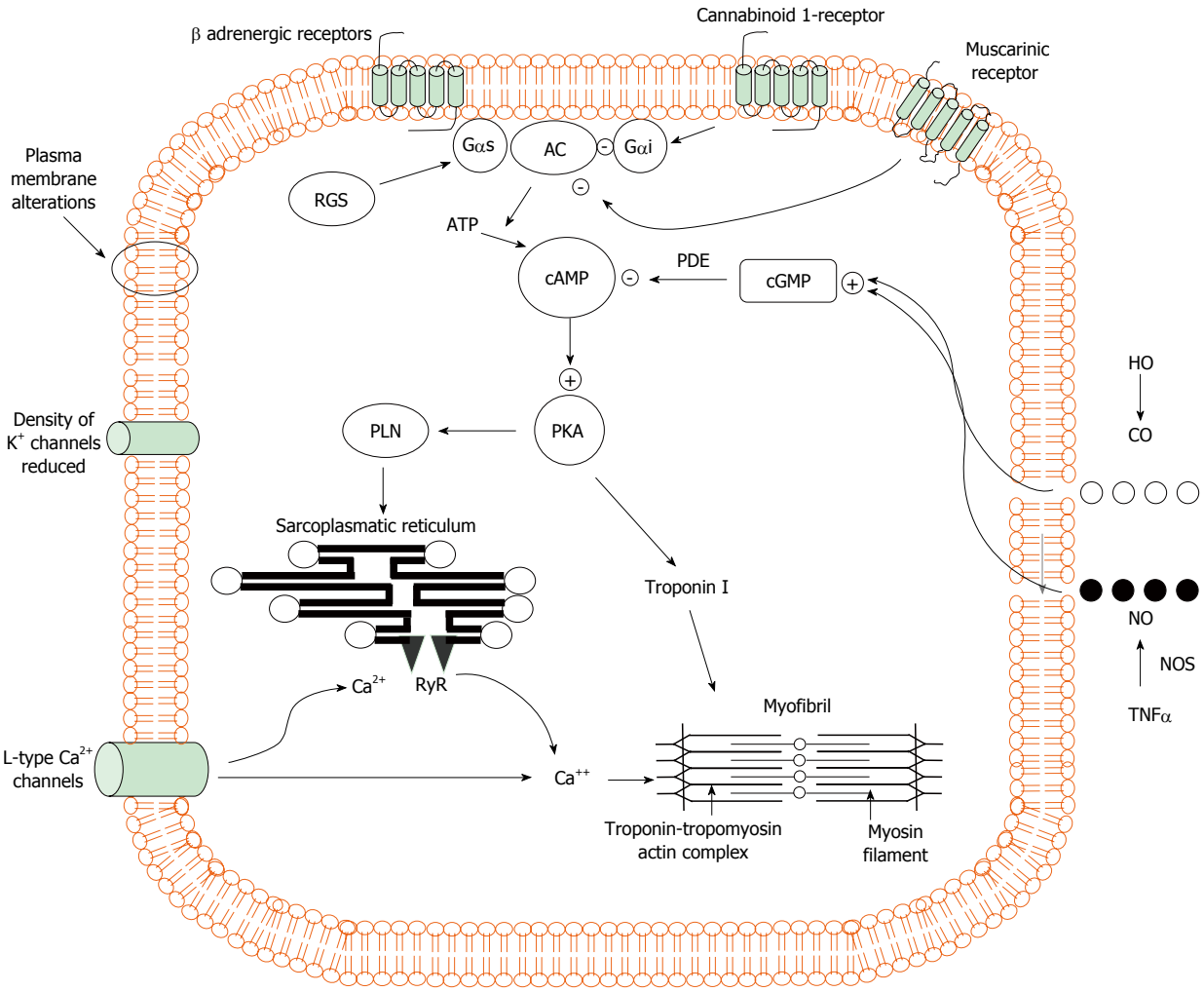

Figure 1 Pathogenic mechanisms of cardiomyocyte contraction in experimental cirrhosis. Cardiomyocytes of cirrhotic animals showed reduced contractility *via* impairment of the βAR signalling (down-regulation, desensitization) which in turn leads to a reduction in βAR density, G_s proteins, and AC activity with resultant decreased cAMP generation. This second messenger cAMP, phosphorylates several proteins leading to intracellular calcium fluxes, calcium release and myocyte contraction. Postreceptor signaling through cAMP is severely impaired. In addition, the decreased fluidity of cardiomyocyte membrane interferes with the function of βAR, L-type calcium and potassium channels and impair stimulation of muscarinic acetylcholine receptors. The activation of CB_1 inhibiting L-type calcium channels and AC together with increased NO and CO levels *via* a cGMP-dependent mechanism also contribute to decreased calcium ion influx or release from the sarcoplasmic reticulum with a related fall of calcium ion content and contractility. +: stimulatory influence; -: inhibitory influence. βAR: β-adrenergic receptors; CB_1: Cannabinoid 1-receptor; AC: Adenylcyclase; cAMP: Cyclic adenosine monophosphate; Gαi: Inhibitory G protein; Gαs: Stimulatory G protein; RGS: Regulator of G-protein signaling; PLN: Phosphoslamban; PKA: Protein kinase A; cGMP: Cyclic guanosine monophosphate; PDE: Phosphosdiesterase; RyR: Ryanodine receptor; HO: Haemoxygenase; CO: Carbon monoxide; NO: Nitric oxide; NOS: Nitric oxide synthase; TNFα: Tumour necrosis factor α.

patients attenuates the contractile responses of neonatal rat heart cells. Therefore, in addition to decreased activity of stimulatory pathways, other inhibitory pathways contribute to the decreased contractility of the cardiomyocyte in experimental cirrhosis.

Endocannabinoids (ECS) are bioactive lipid signaling molecules[47]. The ECS system includes arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) wich are elevated in cirrhosis[48-50]. AEA and 2-AG are generated in response to a rise in intracellular calcium[51]. The interaction of AEA with cannabinoid 1 (CB_1) receptors reduces the contractile response to isoprotenerol of papillary muscles in the fibro-cholestatic heart model. This cardiac decreased β-adrenergic responsiveness could be corrected by CB_1 blockade[52,53]. The activation of CB_1 receptors are coupled to the inhibitory G_i protein inhibiting L-type calcium channels[54] and adenylate cyclase[55] with resultant decreased calcium influx into the cytosol of the cardiomyocyte. It has recently been observed that elevated myocardial AEA levels are associated with high plasma tumour necrosis factor alpha (TNFα) in BDL mice[56]; likewise, AEA levels were lower in anti-TNFα-treated BDL. TNFα-mediated myocardial dysfunction in response to endotoxin stimulation is well documented[57]. These experiments suggest that inflammation might be partially responsible for local production of ECS which reduces the contractile function.

Other experimental studies have also revealed that NO produced in cardiomyocytes from increased

inducible nitric oxide synthase (iNOS) activity has a negative inotropic effect. High levels of NO in the BDL cirrhotic rat model have been shown to decrease the normal papillary muscle contractility whereas the administration of the NOS inhibitor L-NMMA (N omega-monomethyl-larginine) has led to improved blunted contractility[58]. Liu *et al*[59] demonstrated that the heart and aorta of cirrhotic rats contained high levels of soluble cyclic guanosine monophosphate (cGMP) as well as iNOS messenger ribonucleic acid. Stimulation of iNOS is possibly related to inflammatory mediators. Serum levels of cytokines are increased in cirrhosis; likewise, it is known that treatment with L-NAME (NG-L-nitro-arginine methyl ester) works by restoring the depressed inotropic effect of isoprenaline in the BDL rat model[59]. An alternative molecular pathway to explain the role of NO in CCM could be the inhibitory effect of nitration[60].

CMO is an endogenously produced short-lived gas *via* metabolism of haem, whose degradation is catalyzed by enzyme haem oxygenase (HO). The inducible HO isoform may stimulate CMO production in cirrhosis through endotoxemia, cytokines or the activation of the SNS. In the BDL cirrhotic rat, protein expression, and total HO activity were significantly upregulated in ventricles of cirrhotic rats compared with sham-operated control hearts[61]. Treatment with zinc protoporphyrin, an inhibitor of HO, significantly inhibits cGMP production which, in turn, reduces CMO levels and leads to an improvement in blunted papillary muscle contractility but not in control[61]. The activation of the NOS/NO and HO/CMO system significantly increases the cGMP contents in BDL-cirrhotic rats[59]. These findings suggest that the cGMP, either by phosphorylating the kinase G protein or by inhibiting production of the ryanodine receptor (Figure 1)[62], inhibits intracellular free calcium fluxes in the cardiomyocyte thus supporting the idea that NO and CMO production play an important role in the reduced contractile response in CCM.

Apoptosis can be important in modulating heart function. The exchange of Na^+/Ca^{2+} ions is essential in maintaining the steady-state intracellular free Ca^{2+} concentration. The abnormalities in the Na^+/Ca^{2+} transfer in cirrhotic patients results in an excess of Ca^{2+} influx leading to cardiomyocyte apoptosis[63] Recent studies have shown that apoptosis plays a causal role in cardiomyopathies with mediators such as ECS, NO, CMO and endogenous opioids[64] contributing to the activation of the apoptosis pathways. A study in BDL-mice suggest that activation of the extrinsic apoptosis is the responsible pathway[65]. The neutralization of the extrinsic apoptotic pathway improved cardiac contractility.

A mechanism of reduced myocardial contractility *via* the nuclear factor jB (NF-jB) in BDL cirrhotic rats has also been reported[66]. Cardiomyocytes of cirrhotic animals showed reduced contractile response to isoproterenol, with increased myocardial levels of NF-jB. NF-jB inhibition resulting in restoration of contractile function[66].

SYSTOLIC DYSFUNCTION

Systolic dysfunction is mostly latent in patients with cirrhosis. Although left ventricular systolic function (LVSF) at rest, assessed by invasively and non invasively methods, are normal in cirrhotic patients[10,67] subtle alterations could be detected under conditions of stress or by using new echocardiographic techniques at rest[68].

Patients with cirrhosis have documented blunted responsiveness to volume and postural challenge, exercise or pharmacological infusion. Contractile dysfunctions are common in pre-ascitic cirrhotic patients; likewise, these patients show increasing end-systolic volumes[69] as a result of sodium loads. This involvement is more important in patients with ascites[5] despite a decrease in both pre-load and afterload. The altered response to active tilt in cirrhotic patients also suggests an impaired myocardial contractility. During 5 min of standing, cirrhotic patients experienced a decrease in the LV end-systolic volume, SVR and cardiac indexes despite marked increments in HR and in the activity of neurohumoral systems[31]. On the other hand, in patients with cirrhosis there is an abnormal LV response during exercise manifested by an increase in CO and ejection fraction (EF) less than expected in relation to normal subjects[6]. Gould *et al*[3] have reported increasing ventricular filling pressures and an unaffected cardiac stroke index in patients undergoing exercise. Kelbaek *et al*[70] also observed LV contractile function and ventricular wall compliance was reduced in cirrhotic patients.Another noninvasive tool to evaluate ventricular contractile performance is the measurement of systolic time intervals. The pre-ejection period/left ventricular ejection time ratio has been seen to increase from baseline after exercise in cirrhotic patients[6].

Finally, several studies have demonstrated blunted cardiac responsiveness to vasoactive drugs. The infusion of angiotensin Ⅱ produced a normalization of SVR and an increase in pulmonary wedge capillary pressure (PWCP) but not an increase in CO[71]. Similar effects have been produced with the infusion of terlipressin[72]. These results suggest that the normalization of the afterload may detect a LV dysfunction at rest. Stimulation by b-adrenergic agonists reduces the inotropic and chronotropic responses of the heart in cirrhotic patients. Furthermore, administration of dobutamine, a β1-adrenergic receptor agonist, causes only a slight increase in stroke volume and the dose of isoproterenol needed to increase the HR is higher in cirrhotic patients than in normal subjects[73,74]. Other researches[75-77] have also documented this impaired contractile response in experimental cirrhotic models.

New echocardiographic techniques may identify

patients with subclinical ventricular dysfunction more accurately than conventional methods. Two-dimensional speckle-tracking echocardiography (2D-STE) is a novel imaging technique that allows the assessment of LV regional myocardium and global strain in 3 orthogonal directions (longitudinal, circumferential, and radial)[78] by tracking natural acoustic markers (speckles) in a frame-to-frame basis within the cardiac cycle. 2D-STE is less likely to be preload or afterload dependent as compared with standard echocardiographic measures. Nazar *et al*[79] using 2D-STE, found no differences in systolic function in cirrhotic patients with different grades of LVDD. However, there was no a control group. Recently, Sampaio *et al*[80] and Altekin *et al*[81] found that patients with cirrhosis had reduced longitudinal LVSF, despite still having normal EF.

DIASTOLIC DYSFUNCTION

Abnormalities of diastolic function are an early marker of CCM. Patients with CCM display frequently LVDD. The mechanisms underlying the development of diastolic dysfunction remain unclear. Defects in the passive tension of myofilament proteins as well as impaired myocardial relaxation, possibly related to abnormalities in calcium exchange through the sarcoplasmic reticulum, may play a role in the pathogenesis of LVDD. The sarcomere is made up of filaments of various sizes and contains titin. Titin is responsible for the elasticity of the relaxed striated muscle and thus an important determinant of diastolic stiffness in cardiomyocytes. Additionally, diseases that alter diastolic function also alter the myocardial extracellular matrix. In BDL animals, has been observed a decrease in PKA which can reduce phosphorylation of titin and increase passive tension. In addition, the levels of collagen-Ⅰ mRNA in the BDL group were significantly higher than in the sham animals contributing to the pathogenesis of diastolic function[82].

There has been some data indicating that salt retention may play a part in the development of LVDD. Animal models have shown that high salt intake can lead to concentric LV hypertrophy through the activation of cardiac aldosterone production[83]. Aldosterone plays an important role in promoting fibrosis by stimulating fibroblast proliferation and collagen synthesis, triggering proinflammatory factors which lead to the activation of matrix metalloproteinases and over expression of transforming growth factor-β1[84]. LVDD in the CCM results are most likely a result of LV hypertrophy[85,86]. Liver cirrhosis can lead to heart wall thickness changes[87]. We observed that 75% patients with LVDD and cirrhosis had cardiac hypertrophy[10]. Additionaly, cardiomyocyte hypertrophy in cirrhosis with portal hypertension may be an adaptive response to loading produced by the hyperdynamic circulation and the trophic effects of several neurohumoral systems. The fibrosis may be secondary to increased collagen synthesis by stimulation of the RAAS and SNS[88]. In fact, LV hypertrophy[14,67,89] is more common in patients with ascites combined with increasing plasma renin activity (PRA) and norepinephrine plasma levels than in patients with ascites, but having normal PRA levels, or those not presenting ascites[10] at all.

The diastolic dysfunction increase the blood volume in the left atrium (LA) which, in turn, leads to augment the transmitral pressure gradient. Diagnostic evidence of LVDD can be obtained invasively, (LV end-diastolic pressure > 16 mmHg or PCWP > 12 mmHg) or non-invasively by echocardiography. PCWP or mean LA pressure are normal in patients with CCM but there is a significant progressive increase of cardiopulmonary pressures in relation to the degree of LVDD[10,79]. This backward increase in cardiopulmonary pressures is probably related to the lower afterload/central hypovolemia of cirrhosis[90]. Doppler echocardiography has become the noninvasive technique of choice for the assessment of diastolic function. LVDD is characterized by a change in the transmitral blood flow with an increased atrial contribution to the late ventricular filling[91]. Patients with cirrhosis show dilatation and increased LA volumes, increases in LV diameters but not volumes, increases in the thickness of the posterior wall of the LV and the interventricular septum, a prolongation of the isovolumic relaxation time (IVRT), decreased peak E velocity (early rapid filling phase), prolongation deceleration times (DT) of the E wave, and finally peak A velocity increased (atrial contraction during late diastole)[8,69,92]. IVRT and DT may be prolonged in cirrhotic patients irrespective of the presence of ascites but a significantly reduced E/A ratio has been seen in ascitic subjects[67,69]. Traditionally, LVDD is divided into three different filling patterns: normal, pseudonormal, and restrictive. However, conventional Doppler echocardiographic indices (E/A ratio) have clear limitations (age and load conditions)[91,93] and rarely allow for the accurate differentiation between normal and pseudonormal LV diastolic pattern. TDI can overcome some of these factors. The tissue velocity recorded at the basal and septal corner of the annulus mitral (e') is a more sensitive parameter for abnormal myocardial relaxation than mitral variables. TDI velocities have demonstrated a significant correlation with invasive indices of LV relaxation and minimal effect of preload in the setting of impaired relaxation[94]. The American Society of Echocardiography has suggested that LVDD is characterized by the presence of septal e' < 8 cm/s, lateral e' < 10 cm/s, mitral inflow patterns and LA volume index (LAVI) $\geqslant$ 34 mL/m^2. The degree of severity can be graded according to average E/e' ratio. The prevalence of LVDD is relatively high in patients with cirrhosis (43%-70%) despite a normal EF[95,96] and is not related to the etiology of liver disease[97]. However, a variety of comorbid conditions have been associated with development of LVDD[92,98] Therefore,

these patients should be excluded in the assessment of the prevalence of this condition in cirrhosis. Patients with tense ascites show an improvement in LVDD after paracentesis although LVDD in these patients is still abnormal as compared with healthy controls[67]. It has been suggested LVDD contributes to the pathogenesis of fluid retention in these patients[69]. LVDD in most cases is found to be generally mild (grade Ⅰ) or moderate (grade Ⅱ) in severity[10,79,92]. In addition, in patients with CCM there is a relationship between the severity of LVDD and the impairment in LVSF (CO and LV stroke work) and cardiac chronotropic function at rest[10]. Patients with grade Ⅱ of LVDD had a high MELD score[10]. Whereas some studies[99] have reported a correlation between CCM and the severity of liver failure, other researchers[92] have concluded that the cardiac abnormalities were not different between patients with different degrees of liver function. LVDD seems to be independently associated with the presence of bacterial endotoxin. Cirrhotic patients have elevated levels of endotoxemia due to bacterial translocation[100]. A recent study by Karagiannakis *et al*[101] showed that the severity of LVDD determined by the E/e' ratio correlated with the serum levels of lipopolysaccharide-binding protein, a marker of exposure to bacterial endotoxin.

ELECTROPHYSIOLOGICAL ABNORMALITIES

Several electrophysiological abnormalities are found in cirrhotic patients. These include electrocardiographic QT interval prolongation, electromechanical dyssynchrony and chronotropic incompetence.

QT-Interval prolongation

Prolongation of the QT interval (> 440 s) is found in noncirrhotic patients with portal hypertension and in 30%-60% of cirrhotic patients according to the severity of liver dysfunction[102] (Figure 2). QT interval is affected by HR and therefore must be expressed as a rate-corrected (QTc) interval. A Fridericia or specific QT correction formula has been proposed to better account for the contribution of changes in QT-interval in cirrhosis[103]. The QT interval represents the depolarization and repolarization of the ventricles. Prolongation of the QT interval is caused by an increase in action potential duration in, at least, some of the ventricular myocardium cells. At the cellular level, the electrocardiographically prolonged QT interval is primarily based on a reduction of ion channel currents in cardiac plasma membranes resulting in a prolonged repolarisation[104]. The mechanisms underlying prolonged QTc interval in patients with liver disease are not clear; they are thought to be associated, at least in part, with autonomic dysfunction[105,106], and heart exposure to humoral factors (cytokines, endotoxins, and bile salts) through porto-systemic shunts[107-109] in the setting of decreased function of two types of potassium channels in ventricular myocytes[102-104]. Bernardi *et al*[102] found that the prolonged QT interval correlated with circulating plasma noradrenaline which suggests that enhanced sympatho-adrenergic abnormalities are implicated in its pathophysiology. The clinical relevance of long QT in cirrhosis is not fully understood, yet. It is postulated that this alteration is associated with a poorer survival rate in class A patients of Child-Pugh classification[102]. However, other studies have been unable to confirm these relationship[110]. A prolonged QTc interval may be reduced during chronic β-blockade treatment[111] but β-blockers have deleterious effects in patients with cirrhosis and refractory ascites[112-115]. The QTc interval corrects itself in only 50% of subjects after liver transplant (LT)[110,116]. In addition, a prolonged QTc interval (⩾ 500 ms) is frequently observed throughout the procedure of LT, even among patients with baseline QTc < 440 ms[117]. Transjugular Intrahepatic Portosystemic Shunt (TIPS) insertion[108,112] and gastrointestinal bleeding[118] has been shown to prolong the QT interval. There is also clinical evidence that some drugs[119] should be avoided as far as possible during TIPS insertion or LT. Patients with cirrhosis and prolonged QTc interval are at risk of developing ventricular arrhythmias such as torsades de pointes. The risk of development of the latter is unknown but is thought to be rare. Studies on the dispersion of QT interval (the difference between the maximum and the minimum of the QT intervals measured) in patients with liver cirrhosis report a normal variation. Patients with cirrhosis maintain the normal QT-interval diurnal variation between day and night times[120].

Electromechanical uncoupling

Electro-mechanical systole represents the duration of total systolic time interval and has two major components: the pre-ejection and the LV ejection phases. The pre-ejection period is the interval from the onset of ventricular depolarization to the beginning of the LV ejection. The LV ejection time is the systole phase during which blood is ejected into the arterial system. The systolic time interval is dependent on four key factors, namely the heart rate, the preload, the afterload and the myocardial inotropic state. Whereas in normal subjects, the time between the onset of electrical and mechanical systole is normally tightly controlled, it shows a big variability in cirrhotic patients. A disruption in electromechanical coupling leads to the dyssynchrony between electrical and mechanical systole. Studies evaluating cardiac function in cirrhosis at rest and after isometric exercise have reported a electromechanical delay and extended pre-ejection times, thus suggesting that it was a defect in the electromechanical coupling[5]. A functional electromechanical uncoupling has been confirmed in patients with cirrhosis and prolonged QTc interval who showed that the electrical systole was longer than the mechanical systole with the latter being normal[121].

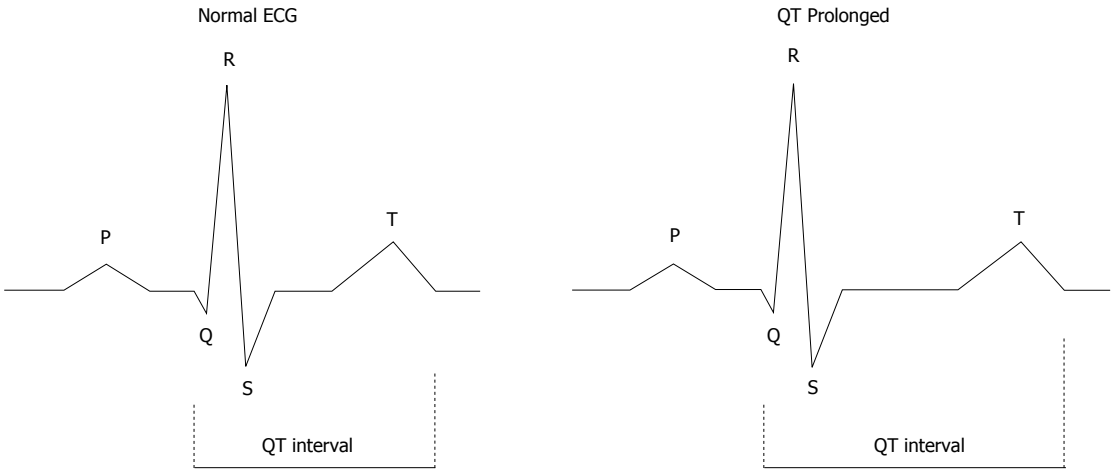

Figure 2 The QT interval. The QT interval is a measure of the time between the start of the Q wave and the end of the T wave in the heart during electrical cycle. The QT interval is dependent on the heart rate and needs to be corrected by heart rate. The corrected QT interval (QTc) estimates the QT interval at a heart rate of 60 bpm. The standard clinical correction in cirrhosis uses Fredericia's formula: $QTc = QT/RR^{1/3}$. The RR interval is given in seconds (RR interval = 60/heart rate). QTc is prolonged if > 450 ms in men or > 470 ms in women. QTc interval prolongation is due to altered repolarization.

The clinical significance of these findings remains unclear. The underlying mechanisms may be related to decreased density of the L-type calcium channel in cardiomyocytes[122]. In addition, an impaired response to the adrenergic drive may affect the excitation-contraction coupling in some patients with cirrhosis.

Chronotropic and Inotropic incompetence

Chronotropic incompetence (CI) is defined as the heart inability to proportionally increase HR in response to metabolic demand. Patients with early stage cirrhosis exhibit responses to dobutamine normal[123]. However, a blunted LV response to dobutamine was observed in 18 of 71 cirrhotic patients with normal LV chamber size and EF[124]. Other studies in patients with cirrhosis have demonstrated CI in response to exercise, tilting, paracentesis, infections and pharmacological stimuli[125-127]. CI is common in patients with cirrhosis regardless of its cause[128,129] and there is more evidence of contractile dysfunction in patients with ascites despite a decrease in afterload. Recently, we have observed that the cardiac chronotropic function, estimated as the HR/plasma noradrenaline ratio, was significantly reduced in patients with grade Ⅱ LVDD when compared to those without LVDD[10] indicating there is CI toward effective arterial blood volume[79,130]. The main cause of CI in patients without structural heart disease seems to be related to SNS activation which is not well translated into HR increases. SNS activation is the likely cause of the down-regulation of βAR, leading to post-synaptic desensitization of the βAR pathway in the sinoatrial node[74,76].

BIOMARKERS OF CARDIAC DYSFUNCTION IN LIVER DISEASE

Two types of molecular biomarkers, have been studied as markers of LV dysfunction in patients with cirrhosis. First, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) measurements are related to several indexes of systolic and diastolic functions. The ANP is predominantly synthesized and secreted in the cardiac atria as a result of direct wall stress; its levels are elevated in patients with increased intravascular volume and LV hypertrophy. The presence of a large LA, as determined by an echocardiography, is considered an indirect marker of filling pressure[131]. Plasma levels of ANP are increased in patients with cirrhosis and ascites[132,133], but in only some pre-ascitic patients[134].

BNP and its prohormone (NT-proBNP) are secreted by heart ventricles in response to massive stretching of muscle cells or to mild cardiac damage. Previous studies in cirrhotic patients have demonstrated that BNP and NT-proBNP serum levels are significantly elevated and correlate with parameters of cirrhosis severity, abnormal cardiac structure (septal thickness and LV diameter at the end of diastole)[135] and function (HR and QT interval) but not with those of hyperdynamic circulation[136]. However, the highest BNP level correlations are seen with end diastolic pressures, hence suggesting that the diastolic stretch is one of the major determinants of BNP induction[137]. Furthermore, the BNP levels of patients with cirrhosis and normal LVSF at rest are also correlated with PCWP values and E/e' ratios[10]. It is recommended that patients with NT-proBNP levels exceeding 290 pg/mL undergo further cardiac evaluation[138].

Second, Troponin is a structural protein composed of three distinct gene products: troponin C, cardiac troponin Ⅰ and T (cTnT). Troponins are specific markers for myocardial injury, specially cTnT, when cardiac lesions are small. Troponin Ⅰ levels are increased in some patients with alcoholic cirrhosis and their concentrations are associated with both lower stroke-volume indexes and LV mass but not related to the severity of cirrhosis and the degree of

portal hypertension[139]. Recently, Wiese *et al*[140] have observed that high circulating levels of cTnT in cirrhosis are correlated with indicators of disease severity and mortality.

Finally, various proteins with enzymatic activities, amongst which myeloperoxidase, galectin-3[141] and copeptin[142] are included, have been investigated in cardiovascular disease. The analysis of these cardiac biomarkers may also provide useful insights in the study of cirrhosis.

Although the prevalence of cardiomyopathy is more frequent and pronounced in patients with advanced liver disease, no association between standard liver function tests, indicating either compromised hepatic synthesis or the presence of portal hypertension, and cardiac dysfunction has been reported. However, when the severity of liver cirrhosis is evaluated by liver-specific scores (MELD score or the Child-Pugh classification), associations between the degree of LVDD, impaired LV systolic and chronotropic functions and MELD scores have been observed[10]. At least one feature of CCM is present in patients who have reached Child-Pugh > 8 points[143]. Correlations between the severity of liver disease and the presence of changes in electrocardiogram at rest have also been found[102]. Therefore, patients with advanced liver cirrhosis with high MELD or Child-Pugh scores suggesting the need for further cardiac investigation.

DIAGNOSIS

The diagnosis of CCM is still difficult to determine due to the lack of a specific diagnostic tools. The EF is known to be a marker of LV systolic function. In patients with cirrhosis, cardiomyopathy occurs with normal LVSF as estimated by the EF at rest. However, LV systolic dysfunction in cirrhosis tends to manifest in conditions of stress although the manoeuvres that can bring about the condition have not been standardized yet. A blunted LV response to catecholamine stimulation through dobutamine stress echocardiography has been observed in 25% of cirrhotic patients[123]. These findings indicate that conventional echocardiographic assessment of LV systolic function based on measurement of EF at rest is not a good index of contractility in cirrhotic patients. Recently, 2D-STE has been proposed as an additional marker for systolic function; hence, this method can detect subclinical LV dysfunction at an earlier stage[13]. Cardiac magnetic resonance imaging has also emerged as another non-invasive technique for the measurement of cardiac function by providing a 3-dimensional representation of the structure of the heart. This approach yields the same indices of diastolic function as Doppler echocardiography but with an increased sensitivity and reproducibility. At present, the use of cardiac magnetic resonance for the assessment of diastolic function may only be considered a research tool. Magnetic resonance spectroscopy has the potential to recognise changes in myocardial bioenergetics and metabolism.

Measurements of LVDD are easily obtainable by conventional echocardiography and TDI. The diagnosis of LVDD in most studies performed in cirrhosis has been based on E/A ratios < 1 ratio and prolonged IVRT and DT. A prolonged mitral DT is an important parameter in drawing conclusions about LV stiffness. Tissue Doppler e' has been demonstrated to decline from normal to LVDD[144]. Most patients with e' (lateral) < 8.5 cm/s or e' (septal) < 8 cm/s and an enlarged left atrium ($\geqslant$ 34 mL/m^2) have impaired myocardial relaxation. The E (mitral)/e' (annular) ratio has been found to correlate well with increased PCWP. There is evidence of LVDD when E/e' ratio is < 8 and E/e' > 15. Therefore, the E/e' ratio and other measurements such as the E/A index and DT are essential for an approach to grade diastolic dysfunction. Prolonged QTc-interval might be helpful to identify patients at risk of CCM which could be diagnosed using a combination of echocardiography and electrocardiogram data (Figure 3). On the other hand, serum markers, such as BNP do not appear to be a sensitive marker for the assessment of subclinical LVDD.

CLINICAL FEATURES

CCM is a subclinical entity. In this regard, when the cardiac function is explored an abnormal ventricular behaviour can be observed. A clinical symptom associated with LVDD is a decreased exercise tolerance. This may be due to LVDD has a negative impact on LVSF through its limitation of the Frank-Starling mechanism[145]. Cardiovascular complications are not clinically evident in patients with CCM during the follow-up period. Heart failure does not present clinically probably because of the peripheral vasodilation. However, conditions that rapidly normalize peripheral vascular tone might precipitate heart failure. Overt cardiac failure has been described after TIPS and LT. CCM plays an important role in the pathogenesis of the impairment of effective arterial blood volume and is a sensitive marker of type 1 HRS development in cirrhosis[10].

Cardiac and circulatory dysfunction in cirrhosis

Research on circulatory function in cirrhosis has been focused for many years on the peripheral arterial circulation. However, recent studies indicate that irrespective of the cause of cirrhosis, there is also a cardiac dysfunction that could be of major importance in the deterioration of circulatory and renal function[130,146,147]. At the initial stages of cirrhosis, portal hypertension induces a progressive reduction in splanchnic vascular resistance due to the overproduction of vasodilator molecules[11,148]. Initially, circulatory homeostasis is maintained by the development of a hyperdynamic circulation characterized by high plasma volume, cardiac index

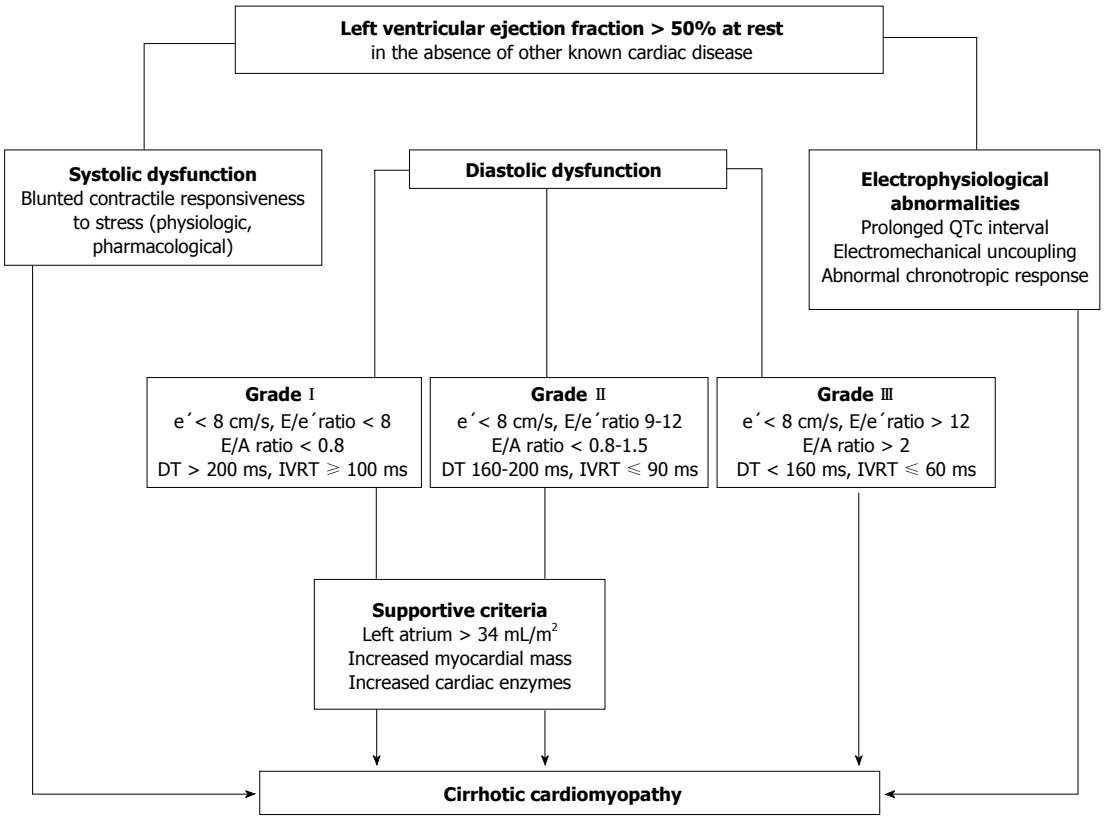

Figure 3 Algorithm for the diagnosis of cirrhotic cardiomyopathy. Three ways to diagnose CCM with normal EF at rest have been displayed: (1) Systolic function. Patients have documented blunted responsiveness to volume and postural challenge, exercise, or pharmacological infusion but the manoeuvres that can bring systolic dysfunction have not been standardized yet; (2) Diastolic function. The diagnosis of LVDD can be obtained by TDI (E/e' > 15). If TDI yields an E/e' ratio (8 < E/e' < 15) other echocardiographic investigations such as blood flow Doppler of mitral valve or pulmonary veins, LV mass index or left atrial volume index are required for diagnostic of LVDD; (3) Electrophysiological abnormalities. a, the prolongation of the electrocardiographic corrected QT interval is common in cirrhosis; b, electromechanical uncoupling is a dyssynchrony between electrical and mechanical systole. The electrical systole is longer in patients with cirrhosis; c, chronotropic incompetence is the inability of the heart to proportionally increase HR in response to stimuli (exercise, tilting, paracentesis, infections and pharmacological agents). CCM: Cirrhotic cardiomyopathy; EF: Ejection fraction; LVDD: Left ventricular diastolic dysfunction; TDI: Tissue Doppler imaging; e': Peak early diastolic velocity at the basal part of the septal and lateral corner of the mitral annulus; E/e'ratio: Peak E-wave transmitral/early diastolic mitral annular velocity; E/A ratio: Early diastolic mitral inflow velocity/late diastolic velocity; DT: Deceleration time; IVRT: Isovolumic relaxation time.

and HR[1,149]. Later, during the course of the disease, increases in the arterial vasodilation in the splanchnic circulation leads to an activation of the RAAS and SNS in order to maintain arterial pressure. However, the progressive decrease in cardiac afterload is not followed by an increase in HR and CO. Several studies suggest that circulatory dysfunction in cirrhosis is associated with a decrease in cardiac function in addition to splanchnic arterial vasodilation. Firstly, chronotropic heart function is progressively and severely impaired in patients with cirrhosis because the HR is unable to keep up with an increasing SNS activity[10,79,130]. Secondly, cardiac dysfunction plays an important role in the pathogenesis of the impairment of effective arterial blood volume in cirrhosis. We have investigated the relationship between cardiac dysfunction and the degree of impairment in effective arterial blood volume (as measured by the PRA) after categorizing the patients into three groups: (1) patients without effective arterial hypovolemia (compensated cirrhosis); (2) patients with ascites and normal PRA (these being a subgroup of patients with early decompensated cirrhosis); and (3) patients with ascites and increased PRA (group with significant effective arterial hypovolemia). Patients with cirrhosis and a maked impairment in effective arterial blood volume showed significantly higher levels of norepinephrine concentration and arterial hypotension as well as lower measurements of LVSF (CO and LV stroke work) and cardiac chronotropic function when compared to those with ascites, but normal PRA, or without ascites (Figure 4)[10]. These patients also showed a higher degree in LVDD which indicates a relationship between the severity of LVDD and other types of cardiac function abnormalities at rest. These findings are an indication that cardiac dysfunction plays an important role in the pathogenesis of the circulatory dysfunction in cirrhosis. In contrast, Nazar *et al*[79] in a recent study have reported that mild LVDD in cirrhotic patients does not correlate with systemic circulatory dysfunction; these findings may be accounted for the fact that only 16% of patients had grade Ⅱ LVDD.

Cardiac and renal dysfunction in cirrhosis

Several studies have showed a clear association between abnormal LVSF and renal failure. A longitudinal

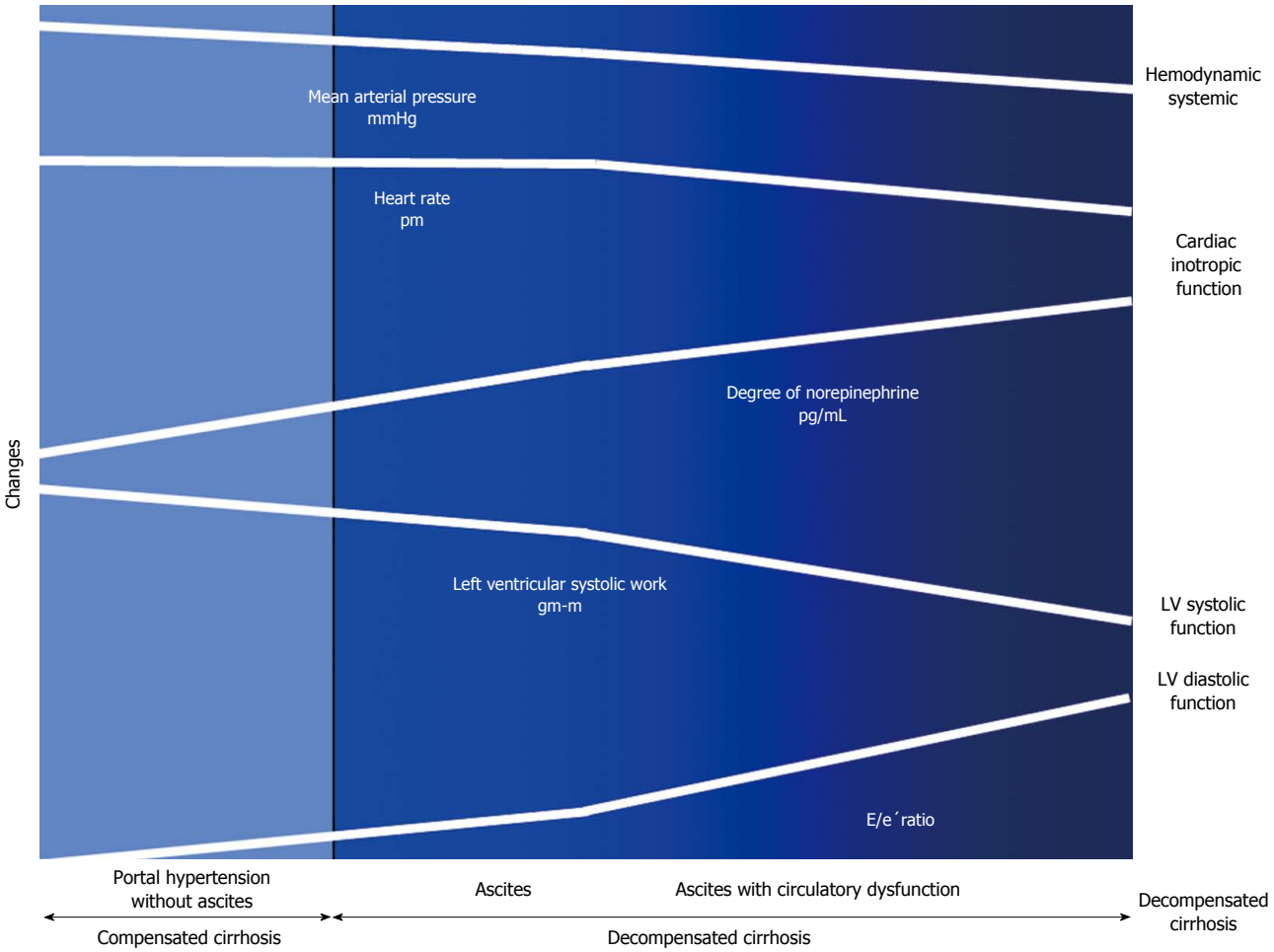

Figure 4 Cardiac and circulatory dysfunction according to presence of compensated and decompensated cirrhosis. There is a progressive splanchnic arterial vasodilatation due to the overproduction of vasodilator molecules. Initially, circulatory homeostasis is maintained by the development of a hyperdynamic circulation. Later during the course of decompensated cirrhosis, patients develop an activation of the vasoconstrictor systems to maintain arterial pressure. In subsequent stages, the progressive decrease in cardiac afterload is not followed by an increase in HR and CO due to a decrease in cardiac function. Finally, in the advanced phase, when impairment of effective arterial blood volume is extreme, patients showed increased activity of NE concentration, arterial hypotension and reduced LVSF (CO and LV stroke work), and cardiac chronotropic function, and a higher degree in LVDD (E/e') as compared to those with ascites, but normal NE, or without ascites. E/e'ratio: Peak E-wave transmitral/Peak early diastolic mitral annular velocity.

study performed in a large cohort of patients with cirrhosis and tense ascites with normal renal function at baseline[130], strongly supports the assumption that CCM contributes to the pathogenesis of hepatorenal syndrome (HRS). These patients were studied at inclusion and following the development of HRS. Forty percent of patients developed HRS. Patients who went on to develop HRS had significantly lower baseline mean arterial pressure, CO, stroke volume (SV), left-ventricular stroke work (LVSW) and significantly higher PRA and norepinephrine concentration when compared with those who did not develop HRS. After developing HRS, patients experienced a further increase in the activity of vasoconstrictor hormones and a further deterioration of their hemodynamics with a decrease in their mean arterial pressure, CO, SV, and LVSW. Baseline PRA and CO were the only independent predictors for the development of HRS. Non-azotemic patients with more advanced stage of CCM could be specially predisposed to develop HRS[10]. Krag *et al*[147] have recently demonstrated a significant relationship between the degree of systolic and renal dysfunction in patients with decompensated cirrhosis. On the other hand, in a different study[146] we have recorded systemic hemodynamics and endogenous vasoactive systems in patients with spontaneous bacterial peritonitis (SBP) at the time of diagnosis and following infection resolution (Table 1). Patients developing HRS after SBP resolution showed a reduction of 32% in CO whereas these changes were not observed in patients who did not develop HRS. SVR values were normal or reduced and the HR did not increase despite an intense stimulation of the endogenous vasoconstrictor systems. Circulatory dysfunction in type-1 HRS, therefore, appeared to be related to the simultaneous occurrence of a progression of arterial vasodilation and a decrease in CO. The results of these studies suggest that LVSF is insufficient to maintain adequate arterial blood pressure and renal perfusion and plays an important role in the pathogenesis of HRS.

Table 1 Changes of cardiovascular function and vasoactive systems from patients with spontaneous bacterial peritonitis to hepatorenal syndrome

	SBP Group-1	SBP Group-2	SBP Group-2	*P* value
	At diagnosis of infection	At diagnosis of infection	At diagnosis of HRS	
Serum creatinine (mg/dL)	1.0 ± 0.3	1.3 ± 0.6	2.5 ± 0.4	< 0.02
MAP (mmHg)	83 ± 10	83 ± 7	73 ± 8	< 0.02
SVR (dyn•s/cm^5)	893 ± 196	1137 ± 220[a]	1268 ± 320	NS
PAP (mmHg)	12.0 ± 2.0	13.2 ± 4.0	12.6 ± 3.7	NS
PCWP (mmHg)	5.9 ± 1.8	5.7 ± 4.0	7.4 ± 2.6	NS
Cardiac output (L/min)	7.4 ± 1.9	5.7 ± 0.9[a]	4.6 ± 0.7	< 0.02
Heart Rate (bpm)	87 ± 16	93 ± 13	87 ± 9	NS
Norepinephrine (pg/mL)	315.7 ± 172	797.3 ± 226.6[a]	1290.5 ± 415.3	< 0.02
PRA (ng/mL•h)	3.9 ± 3.6	18.4 ± 11.2[a]	28.3 ± 12.4	< 0.02

Data obtained from Ruiz-del-Árbol *et al*[146]. Renal failure in SBP is related to a deterioration of circulatory function. At baseline, there were no significant differences between groups in serum creatinine. At diagnosis of infection, patients in the SBP Group-2 showed lower cardiac output and higher levels of systemic vascular resistance, PRA and NE than patients in the SBP Group-1. Following resolution of infection, patients in the SBP Group-2 had severe renal failure and a further decrease in cardiac output, low arterial pressure, and extremely high levels of PRA and NE. There were no significant differences between groups in heart rate and cardiopulmonary pressures. SBP Group-1, Cirrhotic patients with spontaneous bacterial peritonitis that had not developed hepatorenal syndrome in the follow-up; SBP Group-2, Cirrhotic patients with spontaneous bacterial peritonitis that had developed hepatorenal syndrome in the follow-up. [a]$P < 0.025$ *vs* values at diagnosis of infection of SBP Group-1. Data are presented as mean ± SD. SBP: Spontaneous bacterial peritonitis; HRS: Hepatorenal Syndrome; NS: Not significant; MAP: Mean arterial pressure; PAP: Pulmonary artery pressure; PCWP: Pulmonary capillary wedged pressure; SVR: Systemic vascular resistance; PRA: Plasma renin activity.

PROGNOSIS

CCM prognosis is difficult to establish in patients with cirrhosis due to the concomitant liver and cardiac function progressive deterioration. However, one aspect of CCM that has not been specifically addressed is how to assess prognosis in patients with CCM. This information may be particularly relevant to establish priority for patients who are candidates for LT. Most of the existing information on outcome and prognostic factors for these patients is derived from studies before the introduction of the new diagnostic criteria of LVDD. Previous studies have demonstrated that E/A ratio < 1 was an independent predictor of death in patients with cirrhosis who are treated with TIPS[150,151]. Recently, we have observed that the categorization of patients with cirrhosis according to diastolic function has prognostic relevance. Patients with grade Ⅱ LVDD had the shortest probability of survival. Survival was significantly lower in patients with E/e' ratios >10 in the subsequent year[10]. In addition, the accuracy of the E/e' ratio in the prediction of survival was not affected by the severity of liver dysfunction as estimated by MELD. The independency between LVDD severity and other prognostic factors suggests that CCM per se has a negative effect on the natural history of cirrhosis. In contrast, an association between LVDD and bad prognosis could not be found in two recent works[80,152]. A higher prevalence of patients with grade Ⅱ LVDD in our sample (47%) as compared to 16% patients in studies of Nazar *et al*[79] and Sampaio *et al*[152] and a longer follow-up period may explain these differences. In fact, Alexopoulou *et al*[153] observed that patients with mild LVDD had a tendency for worse survival when they were followed up for periods ranging between 15 and 40 mo. A recent study has also showed poorer survival rates in patients with LVDD when they had longer follow-up periods (2 years)[154]. Therefore, patients with severe LVDD might represent a subgroup of the cirrhotic population who are at higher risk of poorer long-term outcomes. This should be taken into account when patients with CCM are listed for LT.

The increased mortality risk in patients with grade Ⅱ LVDD and cirrhosis could be related to a more deteriorated circulatory function that occurs simultaneously with others types of cardiac function abnormalities. These patients with CCM cannot adequately enhanced the ventricular performance of the heart in response to clinical events such as infections. Studies performed in patients with SBP have shown that some patients frequently develop a rapidly progressive deterioration of circulatory function which is thought to be related to the impaired response of the peripheral arterial circulation to endogenous vasoconstrictor systems and a decrease in cardiac output[146]. The impairment in cardiac function occurs because patients with SBP have a deterioration in inotropic and chronotropic function. Such impairment of ejection performance is also recognized in diseases associated with hyperdynamic circulation. Cardiac dysfunction in sepsis and cirrhosis bear remarkable similarities. In both conditions, patients who have an inadequate increase in CO to vascular stress show a higher mortality.

TRANSJUGULAR INTRAHEPATIC PORTO-SYSTEMIC SHUNT

Patients with CCM have reduced ability to compensate for vascular stresses such as TIPS[155]. Transjugular

intrahepatic portosystemic shunt (TIPS) implantation in patients with cirrhosis exacerbates the hyperkinetic circulation and challenges the heart function. These modifications are caused by the shift of a large volume of blood from the splanchnic to the central circulation that occurs after the procedure. Cirrhotic patients show changes of LV diastolic volumes and dimensions, stroke volume[156-158] and a transient pulmonary hypertension[157-159] for 3-6 mo following TIPS implantation but the myocardial thickening continues to increase in the post-TIPS period[37]. A worsening in cardiac hemodynamics already present in cirrhotic patients has been documented when a TIPS is created[160,161]. Clinical episodes of acute pulmonary edema and heart failure have been reported as individual cases[162] as well as in randomised trials in patients with cirrhosis and refractory ascites[163,164]. However, the cardiovascular effects vary to a great extent according to the pre-TIPS state of central blood volume. Patients with effective hypovolaemia show a marked improvement of diastolic function revealed by increasing E/A ratios and a slight reduction of DT[92]. Pre-TIPS LVDD has been associated with a slower mobilization of ascites[149]; it is thought these patients are unable to increase their preload adequately following the TIPS implementation and therefore, the relative underfilling of the effective arterial circulation persists after the intervention. Recently, the studies by Cazzaniga *et al*[151] and Rabie *et al*[150] have demonstrated that the persistence of LVDD one month after the TIPS insertion identifies a new subgroup of patients with a poor prognosis during follow-up. These findings suggest that patients with LVDD should be frequently observed after TIPS implantation.

TREATMENT

Currently, there is no specific treatment for CCM[165]. Atrial fibrillation in patients with CCM it is known precipitate an increase in amount of ascites or dyspnea. Hence the importance of maintaining the sinus rhythm in these patients with CCM.

Pharmacologic therapy

Theoretically, pharmacological agents that facilitate myocardial relaxation and improve LV compliance would be ideal for the treatment of LVDD. β-blockers, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin Ⅱ receptor blockers are the most frequently used agents for treating diastolic dysfunction. ACE inhibitors or angiotensin Ⅱ receptor blockers are probably helpful to reduce the progression of grade-1 LVDD. However, these inhibitors are contraindicated because may precipitate profound hypotension and aggravate the systemic vasodilatory state of patients with advanced cirrhosis.

Positive inotropic agents enhance the rate of LV relaxation but cardiac glycosides are ineffective in increasing cardiac work in patients with alcoholic cirrhosis[166]. Cardiac βAR are down-regulated in cirrhosis, so administration of β-agonists such as isoproterenol or dobutamine are not beneficial for the treatment of LVDD. Since most of the filling of the LV occurs in early diastole, prolonging the diastolic filling time with a β-blocker would not be beneficial in patients with grade-2 LVDD. Animal experimental data has shown that early diastolic relaxation is impaired by β-blockers[167]. In addition, β-blockers may be harmful due to reduction in CO in accordance with a decrease in the HR. In fact, the administration of β-blockers is associated with poor long-term survival in patients with cirrhosis and refractory ascites[114]. These results suggest that β-blockers should be avoided in these patients.

Management of CCM with heart failure should follow the same recommendations as non-cirrhotic patients including salt and fluid restriction, diuretics and afterload reduction[168,169]. The treatment of the loading conditions (preload) should be a goal for the treatment of LVDD. Diuretics are an appropriate therapy for reducing the LV preload. Aldosterone antagonists counteract the effect on fibroblasts and cardiomyocytes growth[170] and reduce the circulatory volume load. Pozzi *et al*[171] have demonstrated that aldosterone blockade by long-term K-Canrenoate administration in Child A cirrhotic patients can lead to decreases in the LV wall thickness; there was no significant change in diastolic function as evaluated by the E/A ratio at 6 mo. These findings may be due to the short duration of the study. The effect of aldosterone antagonists on Child B-C patients is unknown. It is important to note that diuretics in cirrhosis must be used judiciously because of the sensitivity to volume of patients with LVDD bears the risk that excessive diuresis result in a sudden drop of stroke volume.

Cardiomyopathy associated with adrenal insufficiency (AI) is known to cause impairments in myocardial contractility. In addition, relative AI has been reported in approximately 10%-26% of noncritically ill patients with cirrhosis[172-174]. Therefore, AI may contribute to CCM. In such cases, steroid treatment might result in improvement in cardiac function under stressful conditions but this needs further evaluation[175,176].

Improvements in our understanding of the molecular pathogenesis of CCM and its incorporation into the diagnostic and therapeutic approaches will enhance the patient management in this cirrhotic population.

Liver transplantation

Liver transplantation (LT) carries the risk of perioperative hemodynamic impairment. During graft reperfusion there is a hemodynamic stress which is characterized by a sudden increase in pre-load. A retrospective analysis has shown that 23% of cirrhotic patients undergoing LT had a decrease in stroke work despite increases in PWCP during the procedure

after reperfusion[177]. Baseline data of LV systolic or diastolic function could not identify the abnormal heart response during LT. Another retrospective study has reported latent myocardial dysfunction in 35.7% of liver recipients after graft reperfusion[178]. In the setting of a cardiomyopathy, elevation in PCWP levels carries the risk of post-reperfusion hemodynamic instability. Post-reperfusion syndrome affects 8%-30% of patients intraoperatively. This syndrome is characterized by a decrease in mean arterial pressure of at least 30% for 1 min within the first 5 min with bradycardia after unclamping of the portal vein and liver reperfusion[179].

Heart failure, myocardial infarction, and arrhythmias in the perioperative and postoperative periods after LT have been reported in 25%-70% of patients[180,181]. Other retrospective study indicate that systolic heart failure is significantly more likely to develop post-operatively among patients with elevated pulmonary artery or right-heart pressures pre-operatively[182]. Preoperative evaluation with transthoracic Doppler echocardiography can help identify those LT candidates at greatest risk of developing clinical heart failure syndrome postoperatively[183].

Cardiac causes of immediate deaths after LT include post-reperfusion syndrome, pulmonary hypertension and cardiomyopathy[184]. Cardiac reserve pretransplant has been associated with outcomes postoperatively. Nasraway *et al*[185] observed that patients with pre-operative reduced cardiac performance had increased frequency of multiorgan failure and death after LT. These findings in non-survivors could be only explained by an early myocardial depression (within 12 h) postoperatively.

CCM has been evaluated by echocardiography using the E/A ratio in three prospective studies. In the first study[186], all 40 patients showed significantly lower diastolic ventricular function 3 mo after LT which was associated with mild LVH. The second study[187] evaluated 30 patients with repeated measurements in the 13-40 mo following LT. The most striking finding of the study was an increase in the number of patients (60%) with abnormal diastolic function. Both authors comment that these patients did not have cardiac symptoms. Finally, Torregrosa *et al*[188] have demonstrated in 15 patients with repeat measurements 6-12 mo after LT an improvement in cardiovascular changes associated with CCM. On the other hand, in a different study an improvement of QTc interval has been observed at 3 mo following LT in about half of cases[106].

The presence of pre-operative CCM could be a risk factor for complications after LT. Recently, it has been suggested that pre-transplant LVDD is associated with an increased risk of allograft rejection, graft failure and LVH after LT[189,190]. All this data suggests the need for a careful cardiac assessment of cirrhotic patients who are candidates for liver transplantation[191].

CONCLUSION

CCM is a chronic cardiac dysfunction characterized by impaired contractile responsiveness to stress stimuli and/or impaired diastolic relaxation and electrophysiological abnormalities in the absence of other known cardiac diseases. The mechanisms involved in the impaired contractile function of the cardiomyocyte in experimental cirrhosis include impairment of the b-adrenergic receptor signalling, abnormal cardiomyocyte membrane lipid composition and biophysical properties, ion channel defects, and overactivity of humoral inhibitory factors. CCM is a subclinical cardiac failure. Echocardiography allows the detection of LVDD, with the E/e' ratio being the best screening test to detect the condition. The degree of cardiac dysfunction correlates with liver function and the clinical consequences of major importance are related to deterioration of circulatory function and risk of HRS development during the course of cirrhosis. The severity of cardiac dysfunction may be a sensitive marker for mortality. Treatment is non-specific but LT may normalize the cardiac function.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Inflammatory status in human hepatic cirrhosis

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Abstract

This review focuses on new findings about the inflammatory status involved in the development of human liver cirrhosis induced by the two main causes, hepatitis C virus (HCV) infection and chronic alcohol abuse, avoiding results obtained from animal models. When liver is faced to a persistent and/or intense local damage the maintained inflammatory response gives rise to a progressive replacement of normal hepatic tissue by non-functional fibrotic scar. The imbalance between tissue regeneration and fibrosis will determine the outcome toward health recovery or hepatic cirrhosis. In all cases progression toward liver cirrhosis is caused by a dysregulation of mechanisms that govern the balance between activation/homeostasis of the immune system. Detecting differences between the inflammatory status in HCV-induced *vs* alcohol-induced cirrhosis could be useful to identify specific targets for preventive and therapeutic intervention in each case. Thus, although survival of patients with alcoholic cirrhosis seems to be similar to that of patients with HCV-related cirrhosis (HCV-C), there are important differences in the altered cellular and molecular mechanisms implicated in the progression toward human liver cirrhosis. The predominant features of HCV-C are more related with those that allow viral evasion of the immune defenses, especially although not exclusively, inhibition of interferons secretion, natural killer cells activation and T cell-mediated cytotoxicity. On the contrary, the inflammatory status of alcohol-induced cirrhosis is determined by the combined effect of direct hepatotoxicity of ethanol metabolites and increases of the intestinal permeability, allowing bacteria and bacterial products translocation, into the portal circulation, mesenteric lymph nodes and peritoneal cavity. This phenomenon generates a stronger pro-inflammatory response compared with

HCV-related cirrhosis. Hence, therapeutic intervention in HCV-related cirrhosis must be mainly focused to counteract HCV-immune system evasion, while in the case of alcohol-induced cirrhosis it must try to break the inflammatory loop established at the gut-mesenteric lymph nodes-peritoneal-systemic axis.

Key words: Inflammation; Macrophages; Cirrhosis; Alcohol; Hepatitis C virus; Cytokines; Liver

Core tip: This review focuses on new findings about the inflammatory status involved in the development of human liver cirrhosis, avoiding results obtained from animal models. Liver cirrhosis is induced by the two main causes, infection with hepatitis C virus and chronic alcohol abuse. Detecting differences in the inflammatory status between both cirrhosis etiologies could be useful to identify specific targets for preventive and therapeutic intervention in each case.

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INTRODUCTION

Liver cirrhosis is an end stage hepatic disarrangement characterized by a progressive replacement of the functional hepatic architecture by non-functional fibrotic tissue. Alcoholic liver disease (ALD) and hepatitis C virus (HCV) infection are the two main etiologies of chronic liver diseases leading to cirrhosis and liver-related death in the Western world[1]. Nevertheless, hepatic cirrhosis can be also caused by hepatitis B virus (HBV) infection, hemochromatosis, Wilson´s disease, autoimmune hepatitis, nonalcoholic steatohepatitis associated to diabetes or dislipemy[2]. While the overall death rate caused by cirrhosis has fallen in the last thirty years[3], HCV death rates, closely associated with cirrhosis, have been increasing since the 1990s[4].

Survival of patients with alcoholic cirrhosis (ALC-C) seems to be similar to that of patients with HCV-related cirrhosis (HCV-C). However, it has been reported that the risk to develop ascites is higher in ALC-C, while progression to hepatocellular carcinoma is higher in HCV-C[5]. Most experimental data of ALD are obtained from animal models in stages of mild liver injury (moderate inflammation and steatosis), while severe alcoholic hepatitis (AH) in humans is developed in the phase of cirrhosis associated with severe hepatic failure. Furthermore, extrapolation from murine models to man is not always feasible. Hence, human translational studies are crucial for the development of new therapeutic strategies. It is well established that alcohol withdrawal and brief administration of corticosteroids are critical to ameliorate the survival of patients with AH and ALC-C[6]. However, almost 40% of people with the most severe forms of AH will not improve after that treatment[7].

Clearing HCV infection is difficult owing its high tendency to persist and induce chronic hepatitis C in approximately 75%-80% of infected individuals. For this reason, more research on the key factors required for a successful immune response against HCV is necessary to avoid progression toward hepatic cirrhosis. In this respect, it has been shown that the ability to spontaneously eradicate the virus, as well as the outcome of infection upon treatment with human recombinant interferon (IFN)-α correlate most closely with genetic variations within the region encoding the IFN-λ genes (reviewed by Mihm[8]).

Nevertheless, little is known as for the differences and/or similarities between alcohol- and HCV-induced liver disease at the cellular and molecular levels[9] and, to the best of our knowledge, data on the inflammatory status of ascitic human peritoneal cells are very scarce. Hence, understanding the differences in the immune response mechanisms implicated in the pathogenesis of alcohol- and HCV-induced liver disease will contribute to a better development of individually tailored preventive and therapeutic procedures.

Natural history of liver cirrhosis

Hepatic damage leading to cirrhosis is the result of a complex mechanism involving, from direct toxic effects to a sustained inflammatory process, driving to the death of hepatocytes and liver fibrosis, mediated by secretion of several cytokines. The inflammatory reaction is the coordinated process by which the liver responds to local insults, trying to restore the original structure and hepatic function. However, if the local damage is persistent and/or intense, the maintained inflammatory response gives rise to a gradual replacement of normal hepatic tissue by non-functional fibrotic scar. The imbalance between tissue regeneration and fibrosis will determine the outcome toward health recovery or hepatic cirrhosis. The involvement of the innate immune response in the pathogenesis of liver cirrhosis has been largely described[10]. Secretion of interleukin (IL)-1β and tumor necrosis factor (TNF)-α by hepatic macrophages, which are accumulated in the liver during chronic hepatic inflammation has been reported[11,12]. Increased hepatic and systemic injury is related with a high production of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-17, as well as anti-inflammatory cytokines, IL-10 and transforming growth factor (TGF)-β[13]. Galectin (Gal)-3, a crucial component of the

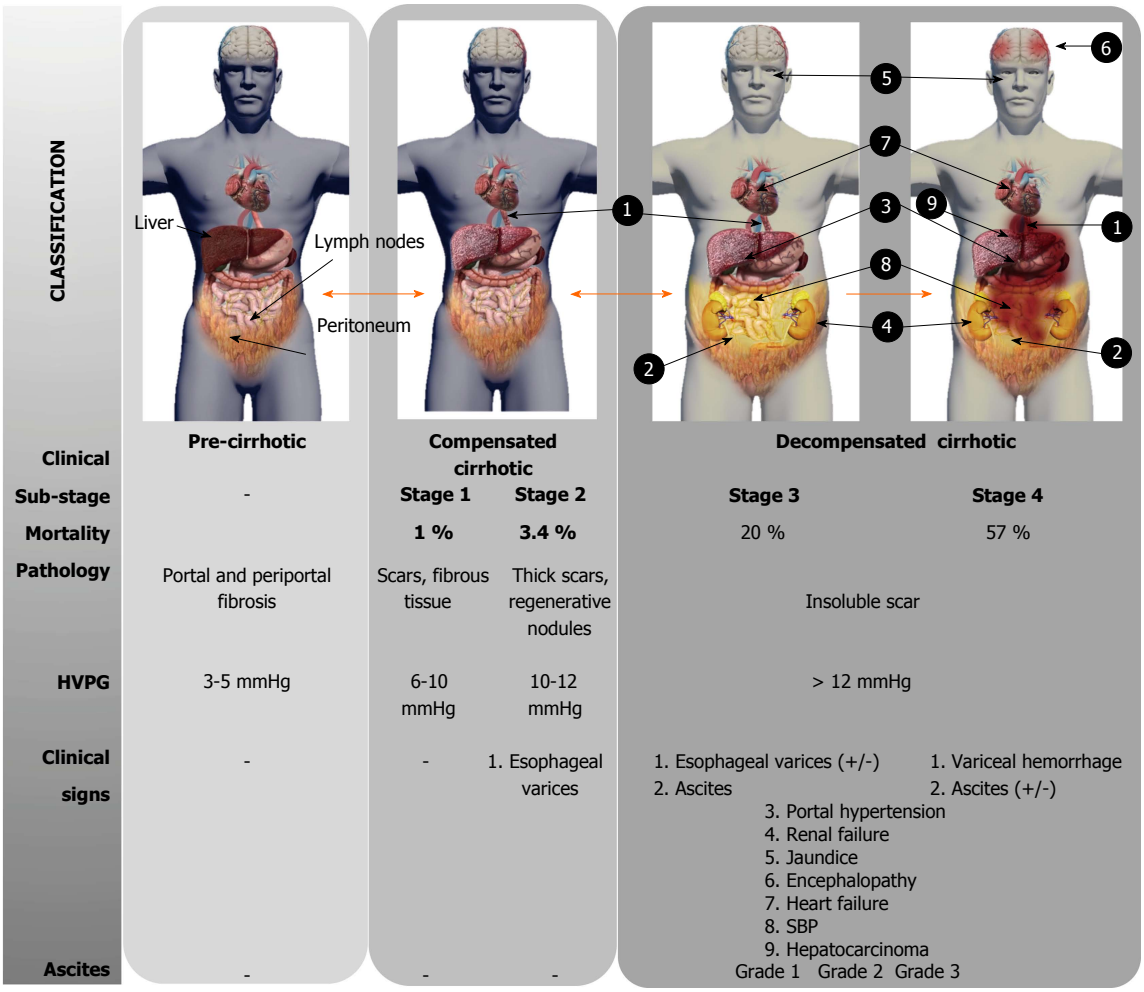

Figure 1 Progression and complications in human liver cirrhosis. The evolution of the liver cirrhotic disease, according to the clinical criteria, mortality rate, pathology characteristic, HVPG, signs and the presence of ascites, is shown. Stage 1 in a compensated cirrhosis differs from stage 2 in the appearance of esophageal varices as well as the increase of portal pressure. Stage 3 is characterized by the presence of ascites and/or esophageal varices. If variceal hemorrhage occurs, cirrhosis gets to stage 4. Cirrhosis could be a reversible process in the earlier stages, as indicated with the orange arrows. HVPG: Hepatic venous pressure gradient; SBP: Spontaneous bacterial peritonitis.

host response in tissue injury and acute inflammation, has also been implicated in the progression toward chronic inflammation after persistent insults. Although normal human hepatocytes do not express Gal-3, it is strongly expressed in the periphery of regenerating nodules in human hepatic cirrhosis produced by a wide range of etiologies[14-16].

Hepatic stellate cells (HSCs) have a fundamental role in liver immunology. These cells are the main storage place for dietary vitamin A, that is required for the suitable function of the immune system, represent a multifaceted source of many soluble immunological active mediators including cytokines and chemokines[17], may function as an antigen presenting cells (APC), and have autophagy capacity. HSCs are crucial sensors of altered liver tissue integrity and able to initiate the activation of cells of the innate immune system[18]. HSC is considered the main fibrogenic cell type in the liver. These cells produce several types of extracellular matrix proteins and, when activated, they lose their Vitamin A and lipids storage compartments and develop a myofibroblastic phenotype[18-20].

Local accumulation of fibrous tissue isolates normal hepatic lobules, hindering the contact and metabolic exchange between hepatocytes and blood sinusoids, disrupting the normal architecture of the liver, which is steadily replaced by abnormal nodules surrounded by fibrous tissue. This process results in a progressive hepatic loss of function, such as catabolism of toxins and drugs, metabolism regulation of carbohydrates, lipids and proteins, synthesis of multiple proteins and others crucial molecules[21]. Although liver cirrhosis conveys a progressive liver derangement leading to the patient's death because limited therapeutic strategies are available, there is increasing evidence suggesting that hepatic fibrosis constitutes a potentially bidirectional dynamic process[22]. Several stages are distinguished according to the clinical progression of liver cirrhosis (Figure 1). Frequently, there is an asymptomatic or oligo-symptomatic stage of variable duration referred to as compensated liver cirrhosis followed by the decompensated liver cirrhosis

stage, characterized by the presence of ascites, hepatic encephalopathy, esophageal varices and bleeding, jaundice, renal and cardiac failure and frequently, hepatocellular carcinoma development[23]. The Child-Turcotte-Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores are currently used to estimate the hepatic dysfunction and define prognosis, but they do not provide direct evidence of the stage or dynamic state of cirrhosis[24].

IMMUNE DYSREGULATION INDUCED BY HCV INFECTION

Liver damage in chronic infection by HCV is currently attributed to host's immune-mediated mechanisms, since HCV itself does not cause a cytopathic effect. Multiple HCV-associated components interact with different elements of the host's innate and adaptive immune system. In this respect, HCV genome RNA, HCV core and other non-structural proteins activate host innate immune response *via* different pathogen-associated pattern recognition receptors (PRRs) such as, Retinoic acid inducible gene-Ⅰ receptor (RIG-I)[25], Toll-like receptors (TLRs)[26,27], Nucleotide-binding oligomerization domain-like receptor family (NLRP3)/caspase-1[28] and protein kinase R[29], expressed on hepatocytes, Kupffer cells, plasmacytoid dendritic cells and natural killer (NK) cells among others. This, triggers specific intracellular signaling pathways leading to secretion of type Ⅰ and type Ⅲ IFNs, pro-inflammatory cytokines, chemokines and other antiviral products aimed to interfere and eliminate the viral infection[30]. However, several HCV-components are able to disrupt and interfere with innate and adaptive cellular components impairing the immunoregulatory and cytotoxic activity of several innate and adaptive cell types, such as APCs, NK cells and $CD4^+$ and $CD8^+$ T lymphocytes, among others. HCV regulation of the cross-talk between hepatic resident and recruited cell subtypes seems to dictate the profile of IFNs as well as pro- and anti-inflammatory cytokines production, which will be critical to determine the outcome of natural and treated HCV-infections.

Monocytes, macrophages and kupffer cells

Myeloid mononuclear cells are integrated by circulating blood monocytes, tissue resident macrophages, referred to as Kupffer cells (KCs) in the liver and dendritic cells. These cell types link innate and adaptive immunity and are crucial in the development and maintenance of many inflammatory diseases. The role of KC in the clearance and persistence of HCV has been recently reviewed by Boltjes *et al*[31]. The number of KC in the liver is increased in chronic HCV infection[32,33], they display an activated phenotype with high mRNA expression levels of CD33 and CD163 receptors[34,35]. Albeit a direct effect of KC on replication of HCV is unknown, it has been described that the TNF-α produced by lipopolysaccharide (LPS)- and HCV-activated KC, enhances HCV infection in hepatoma cells[36]. Furthermore, IL-6, IL-1β, and IFN-β secreted by HCV- or TLR-ligand-stimulated KC pathways[28,37-39] inhibit HCV replication[40,41], suggesting that KC can also exert antiviral effect upon HCV exposure.

Nonetheless, viruses can interfere with the pro-inflammatory activity of macrophages/KC to avoid host defenses. In this respect, several studies have shown that HBV and HCV are able to neutralize the pro-inflammatory activities of immune cells and hepatocytes by interfering with TLR- and RIG-I-signaling[42-46]. However, studies on the effect on human KC are limited, mainly because the difficulty to distinguish between liver infiltrating macrophages and resident KC. Hence, several studies have shown altered TLR responses of blood monocytes obtained from chronic HCV-infected patients, and modulation of cytokine synthesis by HCV proteins[47-49]. Meanwhile, one study described the suppressed effect on the synthesis of type Ⅰ IFN and on the expression of TRAIL in human isolated KC by HCV core protein through disruption of the toll-like receptor 3 (TLR3)/TIR-domain-containing adapter-inducing interferon-β (TRIF)/TANK binding kinase 1 (TBK1)/Interferon regulatory factor 3 (IRF3) pathway[37]. In turn, chronic exposure of APCs to the core antigen induced hyporesponse to TLR ligands through activation of TLR2, which correlated with liver dysfunction as determined by platelet numbers and prothrombin time levels. Furthermore, stimulation with TLR ligands resulted in decreased synthesis of IL-6 by peripheral blood monocytes from HCV- but not from HBV-infected patients[50].

It has also been described that chronic hepatitis C patients have high levels of serum IL-1β in comparison to healthy controls. Several lines of evidence indicate that secretion of IL-1β by resident hepatic macrophages produces liver inflammation through HCV-induced NLRP3/caspase-1 inflammasome signaling. RNA sequencing analysis from the liver of patients with chronic hepatitis C demonstrated that viral engagement of the NLRP3 inflammasome stimulated IL-1β production leading to pro-inflammatory cytokine, chemokine, and immunoregulatory gene expression networks linked with clinical deterioration[28].

NK cells

Relating the role of NK cells in the defense against HCV infection, it is assumed that a strong and rapid NK cell response at early stages of the infection induces strong T cell responses leading likely to HCV clearance. In contrast, chronic HCV infection tends to be related with impaired NK cell activity and phenotypes (reviewed by Ahlenstiel[51]) biased towards impaired ability to produce IFN-γ, elevated expression of NKp46-activating receptor, reduced TRAIL and CD107a expression and impaired degranulation compared with controls[52-54]. Hence, persistence of HCV infection is favored by the

impaired secretion of IFN-γ, which is required to induce a strong T helper 1 (Th1) mediated cytotoxic response. According with this, Li *et al*[55] compared the immune state and correlations between cirrhosis produced by HBV and HCV. The levels of IL-6 (Th2 cytokine) in both groups of cirrhotic patients were increased, while the IFN-γ (Th1 cytokine) was only increased in HBV-related cirrhotic patients. Thus, differences were not detected between these cirrhotic groups except in the IFN-γ level.

Furthermore, it has been described that certain chronically activated NKp46^{high} subsets may be especially active against HSC, a crucial player in liver fibrogenesis. The relevance of NK cells in the resolution of HCV infection can be also exemplified by the effect of genetic polymorphisms of killing inhibitory receptors and their Human leukocyte antigen (HLA) ligands on the individual outcome of HCV infection[56]. Many possible mechanisms, by which HCV may impair NK cell cytotoxic and immunoregulatory functions have been described. Thus, Sène *et al*[57] reported that expression of the Natural killer group 2D receptor (NKG2D) activating receptor on circulating NK cells is down-regulated in chronic HCV infection, although its ligands, MHC class Ⅰ-related chain (MIC) molecules, are expressed on hepatocytes. This impairs NK cell-mediated cytotoxicity and secretion of IFN-γ. Moreover, stimulation of monocytes with HCV recombinant NS5A protein through TLR4 promoted p38- and phosphatidylinositide 3-kinase (PI3K)-dependent IL-10 secretion, while inhibited the production of IL-12. In turn, IL-10 triggered secretion of TGF-β which down-regulated the expression NKG2D on NK cells, impairing their effector activity. Notably, this effect could be impeded by exogenous IL-15, which could be assayed as adjuvant of current treatments.

Moreover, the up-regulation of KLRG1, an inhibitory transmembrane protein expressed on peripheral blood CD56$^+$ NK cells and antigen-experienced T cells in patients with chronic HCV infection, negatively regulated NK cell numbers and functions through the Akt pathway. Blockage of KLRG1 signaling significantly restored the impaired IFN-γ secretion by NK cells from HCV-infected patients[58].

T and B lymphocytes

Besides NK cells, HCV-specific CD8$^+$ cytotoxic T lymphocytes kill HCV-infected cells *via* the perforin/granzyme lytic pathway, but also by secretion of Fas ligand (CD178 or CD95L) and inflammatory cytokines, mainly IFN-γ. In this respect, impaired T-cell responses in chronic HCV-infected patients have been related with HCV persistent infection. Thus, HCV-specific T-cells become unresponsive and apparently disappear in chronic HCV-infections through several possible mechanisms, such as mutations in critical viral epitopes, insufficient help, expansion of regulatory T-cells (Treg) or clonal anergy.

Tacke *et al*[59] reported that HCV induces the accumulation of CD33$^+$ myeloid-derived suppressor cells (MDSC) in human peripheral blood mononuclear cells (PBMC), resulting in reactive oxygen species (ROS)-mediated suppression of T-cell responsiveness. Hence, the accumulation of MDSCs during HCV infection may reinforce and maintain HCV persistent infection[59]. Furthermore, patients with microscopic signs of inflammation displayed a significant higher CD8$^+$ T-cell response to five HCV-derived epitopes from core, NS3A, NS3B, NS4B, and NS5B antigens and also a higher CD4$^+$ Th1 response to the HCV core antigen than individuals with signs of fibrosis/cirrhosis. These results indicate that the insufficient T-cell response to HCV is associated with the progression of cirrhosis during chronic HCV infection[60].

Expansion of CD4$^+$ FoxP3$^+$ Treg in chronic HCV-infected liver has been reported[61]. In this respect, the Tim-3 pathway seems to control the effector/regulatory T cell balance by modifying apoptosis and cell proliferation during HCV infection[62]. In turn, Ji *et al*[63] showed that HCV-infected human hepatocytes express higher levels of TGF-β and Gal-9, and upregulate the expression of both, Tim-3 and regulatory cytokines TGF-β/IL-10 in co-cultured human CD4$^+$ T cells, which evolved into CD25$^+$ FoxP3$^+$ Treg cells. Notably, blockade of Tim-3/Gal-9 ligations abrogated HCV-mediated Treg-cell promotion by HCV-infected hepatocytes, suggesting that interactions of Tim-3/Gal-9 may regulate the development and function of human FoxP3$^+$ Treg cells during HCV infection[63]. More recently, it has been reported that CD4$^+$ T cells expressing HCV-core protein upregulate FoxP3 and IL-10, and suppress CD8$^+$ and CD4$^+$ T cell populations[64].

The role of neutralizing antibodies on the pathogenesis of HCV infections remains unclear. However it is likely that humoral immunity may contribute to the lysis of HCV-infected liver cells *via* antibody-dependent cellular cytotoxicity.

MicroRNAs

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that regulate gene expression by binding to mRNAs to interfere with the process of translation[65]. Several miRNAs are related to different liver diseases depending on the etiologies[66], being miR-122 involved in the HCV replication process. Expression of miR- 16, 193b, 199a, 222, 324 in PBMCs[67] and serum levels of miR-122 and miR-155 are elevated in chronic HCV infection and correlate as inflammatory markers[68].

IMMUNE DYSREGULATION INDUCED BY CHRONIC ALCOHOL ABUSE

ALD is the result of the combined effect of direct hepatotoxicity of ethanol, which is metabolized into acetaldehyde on hepatocytes[69,70] and increases of the intestinal permeability[71], allowing bacteria and

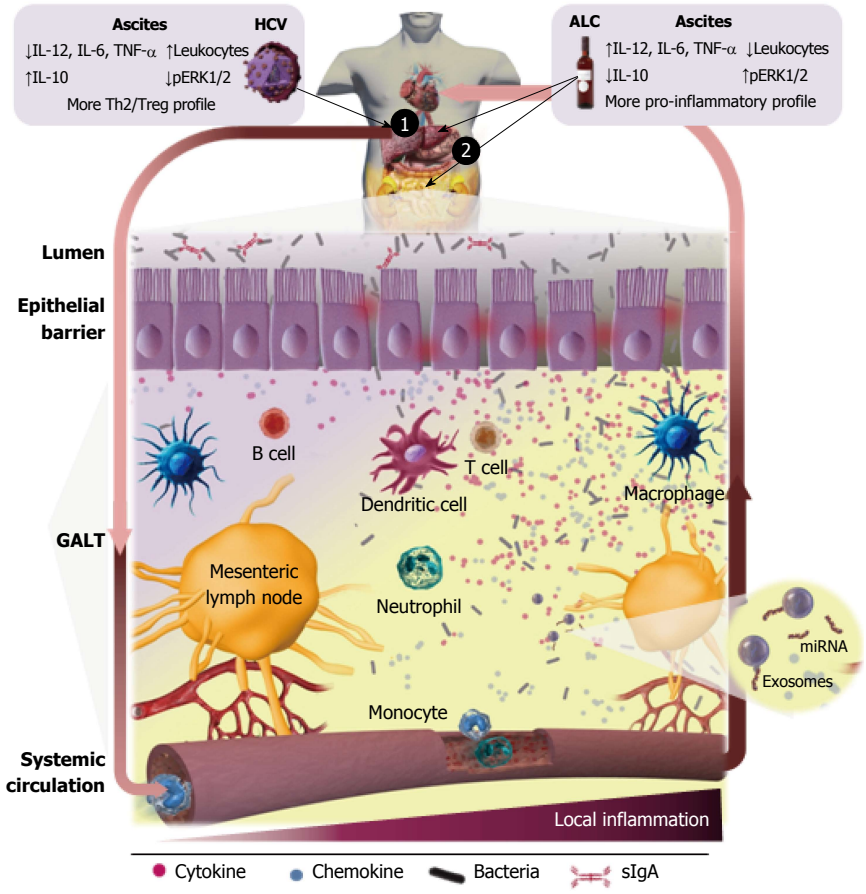

Figure 2 Inflammatory status in alcohol- and hepatitis C virus-induced human liver cirrhosis. The direct injury mediated by the hepatitis C virus infection on the liver (1) and by the toxicity of alcohol simultaneously on the liver (1) and GALT (2), produces an inflammatory response that is spread in the systemic blood circulation. This induces increases in translocation of bacterial products in the earlier stages, and of alive bacteria in advanced stages, which trigger a local inflammatory response within the GALT that further augments intestinal permeability, perpetuating BT. Local abdominal inflammation promotes and maintains systemic inflammation, that would close the loop, aggravating the situation with the progression of the disease, leading to the exhaustion of the immune system defenses. The differences in the inflammatory status between both etiologies are indicated in the figure. HCV: Hepatitis C virus; GALT: Gut associated lymphoid tissue; BT: Bacterial translocation; TNF: Tumor necrosis factor; IL: Interleukin; ALC: Alcohol; Th: T helper; pERK: Phosphorylated extracellular signal-regulated kinase.

gut-derived bacterial products, mainly endotoxin, translocation in the portal circulation (Figure 2). The microbiota composition is altered and correlates with endotoxemia especially in certain subgroup of alcoholics[72,73]. Endotoxin can be frequently detected in serum and ascites of alcoholic patients. Thus, it is widely accepted that gut-derived endotoxins have a prominent role in the induction of liver damage and aggravation of ALD[74]. Indeed, bacterial decontamination and LPS neutralization improves ALD (reviewed by Szabo[75]). The influence of TLR signaling in experimental and human ALD has been analyzed and recently reviewed by Ceccarelli *et al*[76]. Although human clinical evidence of the role of TLR in the liver's inflammatory response in ALD is low, it seems to be in agreement with the experimental data reported in murine models. Stärkel *et al*[77] reported that liver chronic activation of nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), increased expression of TLR3 and TLR7 and elevated secretion of pro-inflammatory cytokines are associated with human end-stage ALD. Related to this, up-regulation of the MyD88-dependent TLR4/NFκB pathway in AH and non-alcoholic steatohepatitis (NASH) in liver biopsies, where Mallory-Denk bodies (MDBs) formed, has been recently described *via* the NFκB-CXCR (C-X-C chemokine receptor) 4/7, providing further insights into the mechanism of MDB formation in human liver diseases[78].

Chronic exposure to bacteria and their pathogen associated molecular patterns (PAMP) activates the inflammatory immune response and produces a dysregulation of the homeostatic mechanisms that control the innate and adaptive immune response, leading to AH, cirrhosis and hepatocellular carcinoma. Acute-on-chronic hepatic failure is characterized by acute worsening of cirrhosis, disturbances in systemic and hepatic hemodynamics, marked activation of the sympathetic nervous system and multi-organ failure, all of them associated with dysregulated inflammation. Hence, inflammation drives the progression of ALD from reversible to advanced stages. These alterations predict high death rates in alcohol-related acute-on-chronic liver failure patients[79]. Among the intracellular

mechanisms trying to counterbalance a dysregulated inflammatory process have emerged autophagy, an important regulatory mechanism of liver homeostasis under physiological and pathological conditions. Thus, there is increasing evidence that complex pathways of autophagy are involved in liver fibrosis, with pro-fibrogenic activity based on its direct activation of HSC, but also with antifibrogenic effects by its indirect hepatoprotective and anti-inflammatory properties (reviewed by Mallat *et al*[80] and Ding[81]). Recently, up-regulation of metacaspase1 and chaperones related to protein quality control in liver biopsies from alcoholic patients has been reported, indicating that autophagy is active in human alcoholic steatohepatitis[82].

Nowadays, there is accumulating evidence of an abnormal frequency and/or function of every single component of the human immune system implicated in advanced stages of ALD. In fact the majority of studies on the systemic inflammatory status on human cirrhosis have been performed in ALC-C.

Monocytes, macrophages and dendritic cells

There is increasing evidence of alcohol-induced dysfunction of peripheral blood and resident myeloid mononuclear cells. Thus, it has been described that high circulating endotoxin levels induced by alcohol abuse leads to activation of KC, which causes a hypoxia-reoxygenation injury process[83]. Also, the function of macrophage Fc gamma receptors is altered in patients with ALC-C, and this impairment likely contributes to the high frequency of bacterial infections in such patients[84].

Dysregulation of the M1 (classical)/M2 (alternative) macrophage polarized balance becomes apparent as a central mechanism implicated in the pathogenesis of chronic inflammatory diseases. This suggests that strategies impairing M1 macrophage phenotype and/or enhancing the M2 macrophage polarization could protect against intensified inflammation and in this way they could limit tissue injury. Relating subpopulation of macrophages involved in AH, it has been reported a complex interplay between different types of macrophages M1, M2a, M2b, and M2c in liver biopsies expressing a diverse array of molecules and receptors[85], as well as a new M1/M2 balance regulatory mechanism, that relies on the apoptotic activity of M2 KCs towards their M1 counterparts. These data suggest that inducing M1 KC apoptosis mediated by M2 might be a pertinent strategy to control alcohol-induced inflammation and hepatocyte injury[86]. Notably, it has been recently described the regulatory activity of overexpressed miR-27a on activation and polarization of monocytes induced by alcohol. Alcohol binge drinking in healthy people results in higher frequency of $CD16^+$ and $CD68^+$ and M2-type ($CD206^+$, DC-$SIGN^+$-expressing and IL-10-secreting) $CD14^+$ blood monocytes. Overexpression of miR-27a in monocytes increased the secretion of IL-10 *via* activation of the ERK signaling pathway by decreasing the expression of ERK inhibitor sprouty2[87].

Furthermore, it has been also described a defect of TLR2 but not TLR4-mediated innate immune response in monocytes from the blood of individuals with compensated chronic ALD[88]. Acute alcohol challenge of monocytes strongly inhibited the NFκB (involved in TLR signaling) activation through mediators of early (LPS) or late (IL-1, TNF-α) stages of inflammation[89].

Neutrophils

Recruitment and activation of neutrophils constitutes the first line of cell defense against microbial insults, and is highly mediated by local secretion of IL-8 and IL-17, among others. Circulating neutrophils in both, alcoholic with severe bacterial infections[90] and patients with cirrhosis, are often very low, displaying lower phagocytic activity/capacity, which predicts the development of infection, organ dysfunction and increased mortality[91]. Defective neutrophil function in ALC-C is caused by decreased generation of superoxide anion and defects of degranulation. Neutrophils from patients with cirrhosis and acute AH have decreased phagocytosis in the early stages of bacterial challenge, although their capacity for ingestion and killing of bacteria is greater than neutrophils from individuals with cirrhosis alone[92]. Furthermore, a greater resting burst, revealing neutrophil activation, and a lower phagocytic capacity was associated with significant higher risk of infection, organ failure, and mortality[93]. Neutrophil phagocytic dysfunction in stable cirrhosis was related with increased expression of TLR2 and 4 in inflamed peripheral tissues and excessive production of inflammatory mediators[94]. Related to this, it has been shown that plasma from AH patients induced an increment of the oxidative burst, while it decreased the expression of CXCR1+2, and the phagocytic capacity of neutrophils obtained from healthy donors, concomitantly with a higher expression of TLR2, 4, and 9 and depletion of ATP. Moreover, the presence of the endogenous endotoxin scavenger albumin, prevented the detrimental effect of patients' plasma on neutrophil TLR expression, phagocytosis and resting burst[95], which attributes an important role to the presence of circulating LPS and others bacterial products.

NK cells

Although alcohol *per se* produced an increase in the number and cytotoxic capacity of NK-cells[96], their lytic ability is depressed in the stage of alcoholic cirrhosis, supporting the hypothesis that efficiency of immunosurveillance may be depressed in these patients[97,98]. On the other hand, alcohol promotes collagen accumulation, which has been correlated with inhibition of anti-fibrotic activity of NK cells[99]. Furthermore, NK-derived IFN-γ induces HSC cell cycle arrest and apoptosis[100].

T and B cells

To date little is known about the mechanisms by which adaptive immunity might contribute to hepatic

inflammation in ALD. Indeed, activation of the adaptive arm of the immune system may be the consequence of an ongoing alcohol-activated innate immune response. Furthermore, cell damage induced by toxic ethanol metabolites and ROS may produce the breaking of the self-tolerance toward liver components[101]. Regarding the alcohol intake duration, the most relevant findings in peripheral blood were a significant activation of the T-cell population, and specifically of the TCRαβ+ subpopulation, with a higher expression of both HLA-DR and CD11c markers, as well as a significant increment of both, NK cells ($CD3^-/CD56^+$) and cytotoxic T cells $CD56^+$. In addition, a decrease in the total number of B cells and their $CD5^+/CD19^+$ subset was found[96].

Patients with ALD had decreased number of lymphocytes, but only individuals with advanced fibrosis had a significant increase in the $CD4^+/CD8^+$ ratio[102]. Alcoholic patients also display $CD57^+$ T cells expansion, which express significantly higher amounts of cytoplasmic TNF-α and IFN-γ after 6 h of TCR-stimulation than the $CD57^-$ counterparts[103]. Defective *in vitro* proliferative activity of PBMC and impaired or normal production of IL-2, together with an increment of lymphoid cells bearing T activation markers and rIL-2 responsiveness was early reported from ALC-C patients[104,105].

It has been recently described that ALD patients lymphocytes express high levels of the immune inhibitory receptors, PD1 and Tim-3 and their respective ligands, CD274 and Gal-9, which modulate the balance between protective immunity and host immune-mediated damage. These lymphoid cells produce lower levels of IFN-γ and higher IL-10 by chronic endotoxin exposure. Furthermore, these effects can be reversed by blocking PD1 and Tim-3, increasing the antimicrobial activity of T cells and neutrophils[106].

It has been described a sharp reduction in the frequency and absolute number of alcoholics' $CD5^+$ B lymphocytes as well as a decrease in the percentage of $CD5^-$ $CD45RA^{high}$ B cells, leaving many patients with a B cell population that was mostly $CD19^+$ $CD5^-$ $CD45RA^{low}$. This subpopulation is phenotypically analogous to the described IgM-producing $CD5^-$ $CD45RA^{low}$ subset, reported by others, and may be enriched for autoantibody-producing cells[107].

Patients with ALD have antibodies against alcohol dehydrogenase (ADH), as well as ADH-specific T-cell responses, which have been associated with alcohol intake in ALC-C patients. Anti-ADH titers are also associated with disease severity and active alcohol consumption. ADH peptides promoted the secretion of IFN-γ, IL-4, and IL-17 from PBMCs of patients with ALC-C. IL-4 secretion was lower in active drinking *vs* abstinence, while IL-17 production was higher. The intensity of the predominant Th1 responses correlated with disease severity[108].

A prospective study reported an association between the presence of antibodies against alcohol-modified hepatocytes and an augmented risk of developing ALC-C[109]. Furthermore, alcohol abusers with lipid-peroxidation derived antibodies have a five-fold higher presence of increased levels of TNF-α in plasma than heavy drinkers with these antibodies within the normal range[110]. Furthermore, the association of high TNF-α and lipid-peroxidation-induced antibodies raises by 11-fold the risk of progressing toward advanced ALD. Of note, the combination of steatosis and elevated titers of antibodies against lipid peroxidation-derived products is an independent predictor of severe fibrosis/cirrhosis in alcohol-drinking people with chronic hepatitis C[111]. B cells from ALC-C patients produced spontaneously more IgA than healthy controls. Moreover, the production of IgA by oligodeoxynucleotides with CpG motifs-activated B lymphocytes was significantly increased compared to controls. These results strongly suggest that TLR priming of B cells could account in part, for the hyperimmunoglobulinemia detected in ALC-C patients[112], although it is also established that hyperimmunoglobulinemia in chronic hepatic diseases is mostly caused by the collateral circulation secondary to portal hypertension, antigens and endotoxins from gut that bypass the liver and contact with the antibody-producing cells[113].

Cytokines

Alcohol intake activates the innate immune system and induces several pro-inflammatory cytokines such as IL-1β and TNF-α, inducing hepatocellular damage. Although it is well established that mediators of the inflammatory process participate in ALD pathogenesis, the exact contribution of inflammasomes in ALD is still unclear[100]. In this respect, blocking IL-1β activity with recombinant IL-1RA (anakinra) ameliorated alcohol-induced hepatic inflammation and damage. IL-1β gene polymorphisms have been correlated with AH[114]. Furthermore, high expression of IL-18 has been associated with advanced ALD[115]. Besides PAMP, inflammation is also elicited by damage associated molecular patterns (DAMP). It has been recently described that endogenous signals of metabolic danger, such as uric acid and ATP, are implicated in inflammatory cross-talk between hepatocytes and immune cells and have a prominent participation in alcohol-induced liver inflammation[116]. Activation of inflammasomes in liver biopsies from AH patients has been associated with MDBs formation, suggesting that MDB could be indicative of the extent of inflammasome activation[117]. More studies are needed to elucidate the exact alcohol-responsive cells *in vivo* and to clarify the role of inflammasome components associated with ALD.

Human ALD is also associated with the activation of the IL-17 pathway. In AH, liver infiltration with IL-17-secreting cells is a crucial feature that might contribute to liver neutrophil recruitment and it correlates to MELD score in ALD. PBMC of ALD patients produce

higher levels of IL-17, and their $CD4^+$ T cells are highly Th17. The IL-17 receptor is expressed by HSCs, which recruited neutrophils after IL-17 stimulation in a dose-dependent manner through IL-8 and Gro-α secretion *in vitro*[118]. Activation of the innate immune system results in elevation of hepatoprotective mediators such as IL-6[119], and anti-inflammatory cytokines, such as IL-10, which play a relevant role in meliorating alcoholic liver damage and inflammation[120]. However, chronic alcohol intake moderates the signaling pathways triggered by those cytokines, thereby decreasing their anti-inflammatory and hepatoprotective activities, and contributing to progression of ALD[121,122]. Thus, while compensated ALC-C is characterized by serum increases of IL-6 and decreases of IL-10, patients with decompensated ALC-C, besides increased IL-6, have also increased concentrations of TNF-α and IL-8. In turn, TGF-β1 and IL-10 levels were alike to those found in healthy controls. Hence, significant alteration of the balance between pro-inflammatory and anti-inflammatory mediators is characteristic of compensated and especially of decompensated ALC-C[123]. Recently, fibroblast growth factor-inducible 14, a molecule included in the TNF receptor superfamily, was described to be over-expressed in the liver, especially by hepatic progenitors, in patients with AH and it correlated with aggravated stages[124].

Serum IL-12 levels, an important cytokine driving Th1 cell-mediated responses, were higher in AH, ALC-C and alcoholic steatosis patients than in a control group. IL-12 presented good sensitivity and specificity in the diagnosis of ALD[125].

Increased serum levels of activin-A (a component of the TGF superfamily, involved in hepatic fibrosis, regeneration and stem cell differentiation) only in patients with ALC-C or hepatocellular carcinoma suggest its potential role in the pathophysiology of ALD[126].

Up-regulation of the pro- and anti-inflammatory cytokine system and simultaneous desensitization of effector cells in patients with ALC-C, could explain the attenuated systemic inflammatory response to chronic endotoxemia. This dysregulation of the immune state may produce the failure of the host defenses against infections, which are frequent complications of ALC-C[127].

Complement

A recent work has suggested that alcohol drinking increases the activity of the complement system in the liver, contributing to the inflammation-associated pathogenesis of AH[128].

Chemokines

The main neutrophil's chemoattractant, IL-8 is activated in ALD, especially in AH, and it correlated with liver damage. The levels of IL-8 can reflect the stage and severity of ALD, and may predict the survival of patients with AH[129,130]. Liver samples from AH show up-regulated expression of the CXC subfamily members IL-8, Gro-α, CXCL5, CXCL6, CXCL10, and platelet factor 4 and correlate with neutrophils infiltration. The expression of CC chemokine CCL2 (C-C ligand-2), but not CCL5, is also increased. Higher expression of IL-8, CXCL5, Gro-γ, and CXCL6 levels are related with worse prognosis[131]. On the other hand, CXCL5 levels are lower in the plasma of people with chronic liver disease, which suggests that CXCL5 might be implicated in its pathogenesis[132,133]. The CXCL9 (ligand of CXCR3) has also effects on the liver, in fact it is released by the liver and might contribute to hepatic and extrahepatic organ malfunction. High levels of CXCL9 are associated with higher mortality in cirrhotic patients with severe portal hypertension receiving transjugular intrahepatic portosystemic shunt[134]. A recent study has described that elevated serum levels of CXCL1, mostly expressed by mononuclear cells activated by LPS, together with a polymorphism in the gene encoding for this molecule are risk factors for ALC-C[135].

CCL20 is a strong chemotactic factor for lymphocytes that binds to the chemokine receptor CCR6. Hepatic and serum levels of CCL20 are elevated in people with AH and correlated with the grade of fibrosis, endotoxemia, portal hypertension, disease severity scores and short term mortality[136].

MiRNA

Several members of the miRNAs family are affected by the presence of alcohol, producing an abnormal miRNA profile in the liver and peripheral blood in ALD. The functions and advances on the effect of circulating miRNAs in human alcoholic diseases, mainly focusing on inflammation and cell survival after ethanol/LPS treatment, have been reviewed by McDaniel *et al*[137], Szabo[75] and Gao and Bataller[138].

Other molecules

Systemic blood, portal and hepatic levels of Gal-3 are increased in patients with ALC-C and it has been described to be negatively associated with liver function[139].

A role for osteopontin in AH has been described. Hepatic expression and serum levels of osteopontin are highly elevated in AH compared to normal livers and other classes of chronic liver diseases, and correlated with short-term survival[140].

STUDIES COMPARING HCV- *VS* ALCOHOL-INDUCED CIRRHOSIS

A comprehensive transcriptional study using oligonucleotide microarrays on hepatic biopsies from cirrhotic patients evidenced that there are genes differentially expressed between ALC-C and HCV-C. Various of the gene expression changes detected in

the HCV-induced cirrhotic livers were associated with the activation of the innate antiviral immune response, while differences between chronic liver injury due to HCV or ethanol seem to be more associated with the regulation of lipid metabolism and deposition of extracellular matrix components concomitantly with others related with macrophage activation[1,3,4,9,141]. When severity, measured by CTP classification, of cirrhosis from each etiology was compared, a different gene expression pattern was identified in clinical stages of ALC-C[9], but not in HCV-induced disease. Thus, CTP class A ALC-C livers displayed unique expression patterns for genes involved in the inflammatory response, including those acting on macrophage activation and migration, as well as oxidative stress and lipid metabolism. This is in agreement with the above mentioned studies demonstrating that the exposition to alcohol activates the innate immune response and induces several pro-inflammatory, hepatoprotective and anti-inflammatory cytokines[142]. However, chronic alcohol intake diminishes the intracellular signaling cascades induced by several cytokines, decreasing their anti-inflammatory and hepatoprotective actions, and then, contributing to the development of ALD[122].

Transcriptome studies of human liver samples has revealed that the expression of several TNF superfamily receptors is up-regulated in the liver of patients with AH compared with healthy individuals or patients with other hepatic diseases. Among them, Fn14, was the only TNF superfamily molecule exclusively up-regulated in AH compared with other hepatic diseases and correlated with both short-term mortality and portal hypertension severity[124].

Advanced oxidation protein products (AOPP) are oxidative stress markers with a pro-inflammatory activity that accumulate in hepatic cirrhosis. AOPP level positively correlated with the Child-Pugh score in ALC-C but not in HCV-C, and the correlation with the markers of chronic inflammation, more specifically TNF-α, was stronger in ALC-C. This suggested that oxidative stress may be a mediator of chronic inflammatory status at the initial stage of ALC-C. In turn, AOPP in HCV-C was less related with the inflammation, although a significant correlation with antioxidant defenses could be detected[143].

The size of the spleen, recorded by sonography and computed tomography, also varies by the etiology of the cirrhosis, being in the alcohol group significantly smaller than in the hepatitis C and NASH groups[144].

DEVELOPMENT OF ASCITES INDUCED BY LIVER CIRRHOSIS

Ascites is the most common complication of liver cirrhosis. Thus, 60% of patients with compensated cirrhosis develop ascites within 10 years during the course of their disease[145]. As liver fibrosis progresses the presence of regenerative nodules and bioactive molecules increases, driving to portal hypertension. Ascites only appears when portal hypertension and, specifically sinusoidal hypertension, has developed producing arterial splanchnic vasodilatation. This causes an increment of capillary pressure and permeability, and a decreased effective volume of arterial blood. The increase of plasma volume and cardiac output are adaptable mechanisms for this reduction. Activation of both the sympathetic nervous system and renin-angiotensin-aldosterone system induce a compensatory retention of sodium and water, thereby leading to the accumulation of ascites[146]. The ascites is classified on the basis of quantitative criterion and determines the clinical treatment: grade 1 corresponds to mild, only detectable by ultrasound and is not treated, grade 2 corresponds to moderate ascites, evident by slight symmetrical abdominal distension, which is treated by reduction of sodium intake and diuretics, and grade 3 is a large ascites volume with manifest abdominal distension, which is treated by large-volume paracentesis, followed by limitation of sodium intake and diuretics (Figure 1). The presence of ascites in cirrhotic patients usually goes parallel with worsening of the clinical state and predicts a poor prognosis, thus many patients are referred for liver transplantation after development of ascites[147]. Bacterial infections are frequent complications appearing in patients with cirrhosis and ascites, being spontaneous bacterial peritonitis (SBP) the most habitual and clinically relevant[148].

BACTERIAL TRANSLOCATION IN LIVER CIRRHOSIS

Commensal microbiota has defined lines of communication in peaceful coexistence with the host, without induction of pro-inflammatory responses in healthy conditions. Bacterial translocation (BT) defined as displacement of bacteria and/or bacterial components from the gut to the mesenteric lymph nodes (MLNs) (Figure 2) is a crucial physiological process for the host´s immunity training. In contrast, throughout the course of liver cirrhosis there is a "pathological increase of BT"[149] due to changes and overgrowth of intestinal microbiota[150], an increase of intestinal permeability and the dysregulation of the gut associated lymphoid tissue (GALT) immune response (revised by Wiest *et al*[151]). Thus, bacterial products and/or alive bacteria of intestinal origin come across the intestinal wall[152], reaching the MLN and ascitic fluid from where they can be carried by the lymph toward the blood circulatory system, and finally disseminated toward other organs. The dysregulation and deterioration of the immune system mechanisms paralleled the disease progression[10,151].

Significant increases of LPS and LBP have been detected in cirrhotic patients[153]. Bacterial DNA may be

also present by BT in ascites of as many as one-third of cirrhotic patients with non-neutrocytic and culture-negative ascitic fluid[154] and it could be also detected in peripheral blood[155]. On the other hand, and in contrast to that reported in hospitalized patients (30%-40%), bacterial DNA is rarely detected in serum and ascitic fluid of cirrhotic outpatients with non-neutrocytic ascites[156]. Bacterial peptides (glyceraldehyde-3-phosphate dehydrogenase A, Porin OmpC and HSP60) have been also detected in ascitic fluid from a group of patients with advanced cirrhosis and culture-negative ascites[157].

ABDOMINAL INFLAMMATION IN THE ABSENCE OF INCREASED PERMEATION OF VIABLE BACTERIA

In stages of cirrhosis without increased permeation of viable bacteria, the systemic inflammation developed in response to the HCV infection or alcohol abuse described above, induces small but continuous increases in translocation of bacterial products, which trigger an increased pro-inflammatory cytokine response and release of ROS within the GALT in order to improve the anti-bacterial defenses.

The intestinal inflammation in cirrhotic patients could be suggested by the elevated fecal polymorphonuclear leukocytes elastase concentrations[158]. Patients with advanced hepatic cirrhosis, and particularly those with ascites, had augmented local production of TNF-α in MLNs, whereas the levels of IL-6 were not different between cirrhotic and controls. This could be attributed to BT and might contribute to systemic alterations of cirrhosis[159].

The translocation of bacterial DNA triggers an increased pro-inflammatory cytokine response with high levels of TNF-α, IL-2, IL-6, IL-12, increased expression of the complement system proteins C3b, membrane attack complex and C5a[160-162], and release of ROS and nitric oxide (NO) through the inducible form of NO synthase[155]. The translocation of LPS induces the production of TGF-β, IL-6, IL-1β and hyaluronan by peritoneal cells in ascites[163]. The presence of bacterial peptides in ascitic fluid of patients with advanced cirrhosis and culture-negative ascites, is associated with elevated levels of C3b and membrane attack complex proteins and a marked inflammatory response with higher levels of TNF-α and IFN-γ[157].

The peritoneal macrophages from individuals with decompensated cirrhosis and culture-negative ascites, present a predominant activated state[158,164,165]. These cells show a pre-activated immune status at baseline, with high CD54, CD86 and HLA-DR surface marker expression levels, increased phosphorylated levels of ERK1/2, PKB and c-Jun intracellular signaling molecules, and enhanced secretion of IL-6. This fact probably reveals that consecutive episodes of BT boost a sustained immune response in these patients, even in the momentary absence of bacterial products. This primed status would favor an IL-6-controlled rapid response against repeated BT episodes[164]. *In vitro* studies performed with peritoneal macrophages obtained from ascites of cirrhotic patients, have demonstrated that secretion of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 from this clinical situation strongly depends on the MAPK signaling pathways, while the PI3K-Akt (also known as Protein kinase B) route plays a relevant role in the regulation of the anti-inflammatory function mediated by IL-10[166,167]. The inhibitors of mitogen-activated protein kinase kinase (MEK1) and c-Jun N-terminal kinases (JNK) reduced the secretion of IL-1β, IL-6 and TNF-α and could serve as therapeutic agents for pharmaceutical intervention to decrease hepatic damage, reducing the inflammatory response associated to liver failure. On the contrary, PI3K-Akt inhibitors suppressed the production of IL-10, and increased the release of IL-1β, mostly by enhancing the release of intracellular IL-1β and caspase-1 toward the extracellular medium. Therefore, the inhibitors of PI3K-Akt are discarded as potential therapeutic agents in hepatic fibrosis, as these drugs would promote the inflammatory response. Macrophages from non-infected ascitic fluids showed constitutive activation of caspase-1 and a notable increase in the expression of IL-18, IL-1β, and AIM2 compared to blood macrophages. The activation of the inflammasome *in vitro* did not require a priming signal, supporting the pre-activated state of these cells[167,168].

As described above, hepatic cirrhosis patients present an altered B lymphocyte function and increased systemic immunoglobulin levels. However, in spite that the serum[113] and salivary[150] levels of secretory IgA (sIgA) are elevated in these patients, the concentrations of fecal sIgA as well as the secretion of mucosal sIgA into the jejunum are decreased[150,158].

Exosomes are small membrane vesicles released from many cell types which contain functional proteins, mRNAs and miRNAs. Exosomes can be engulfed by other cells modulating their functions and playing an important role in the control of immune responses[169]. Exosomes purified from ascites of hepatic cirrhosis patients, have proved to induce the production of IL-1β, IL-6 and TNF-α through NFκB/STAT3 activation in a TLR-dependent way, after internalization by monocytic cells *in vitro*[170]. Furthermore, increased levels of free miR-212 have been detected in the gut after alcohol consumption, resulting in the inhibition of the expression of the tight junction (TJ) protein ZO-1, increasing the gut permeability[171].

The fibrinolytic activity of plasma from patients with decompensated cirrhosis and ascites is higher than that of patients without ascites. In turn, the ascites from such patients has even higher fibrinolytic activity than their plasma, as suggested by: the lower levels of plasminogen and fibrinogen, the elevated fragment D-dimer and fibrin split levels, and the short euglobulin

lysis time (fibrinolytic activity) found in the ascitic fluid. Since ascites fluid re-enters the systemic circulation *via* the thoracic duct or/and *via* a natural peritoneovenous shunt, ascites can be considered as a pathological fluid that contributes to the systemic fibrinolytic state found in most patients with ascites[172].

The peritoneal immune response differs from the systemic one in non-infected cirrhotic patients with ascites, and could potentially determine the likelihood for future adverse events. Hence, subjects with advanced liver cirrhosis and ascites showed significantly greater levels of IL-2, IL-4, IL-6, IL-8, IL-10, monocyte chemotactic protein (MCP)-1, TNF-α, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in the ascitic fluid compared to serum, while the levels of IL-1α, IL-β and IFN-γ were not statistically different[173].

The local inflammation caused by BT in this cirrhotic stage further loosens intestinal TJ-function augmenting intestinal permeability[174] and perpetuating BT. Alterations in TJ-proteins have been demonstrated in duodenal biopsies with diminished expression of occludin and claudin-1 that progressively increase from crypt to tip of the villi[175,176]. Therefore, under cirrhotic situations, loosening of TJs may allow an increased accessibility of viable bacteria and bacterial components to areas of free passage. As cirrhosis advances, the gut evolved into a major source of activated immune cells and pro-inflammatory cytokines, inducing and maintaining a systemic inflammatory status[10].

ABDOMINAL INFLAMMATION IN THE PRESENCE OF INCREASED PERMEATION OF VIABLE BACTERIA

Progressively, in advanced decompensated cirrhosis a combination of severe impaired functional capacity of the liver, a sustained failure of both the mononuclear phagocytic system and the protein synthesis involved in the immune response is established. Furthermore, the increased intestinal permeability that allows the epithelial crossing of viable bacteria from gut, and the consequent deterioration of the systemic inflammatory status, determine the difficulty of the immune system to solve this situation. Then, it switches into a immunodeficient state, which characterizes severe decompensated cirrhosis with extra-hepatic organ failure stages[10].

Prostaglandin E2 (PGE2), likely produced by circulating monocytes, KC and resident macrophages, has been recently identified as a major contributor to the immunosuppressive status in acutely decompensated cirrhosis. In addition, the decrease of albumin (that reduces the bioavailability of PGE2) levels present in these patients seems to be associated with the increased free PGE2 levels[177].

Ascites from cirrhotic patients presents a reduced opsonic activity as a consequence of reduced concentrations of C3 and C4[178,179]. The concentration of C3 is the main factor to confer local defense against infection of ascitic fluid. Hepatic production of C3 and its concentration in ascites are significantly decreased in patients with advanced cirrhosis, and also in those who develop SBP respect to cirrhotic patients without infection[180]. On the basis of these findings, defects of opsonophagocytic activity seem to contribute in the increased susceptibility to infection in cirrhotic patients[181].

Neutrophil chemotaxis and phagocytosis were decreased in cirrhotic patients with previous episodes of bacterial infection compared with non-infected subjects. The expression of complement receptor 3 (CR3) in neutrophils from peripheral blood was significantly increased in cirrhotic patients, whereas it was significantly reduced in elicited neutrophils of cirrhotic patients with previous bacterial infection[182]. Lower respiratory burst in response to *Escherichia coli* was detected during infection in hepatic cirrhosis[183]. These data suggest that impaired neutrophils recruitment to the infection site caused by BT and defective phagocytic activity may contribute to bacterial infections in cirrhotic patients with advanced liver disease.

The ascitic NO concentrations registered at diagnosis of an infectious episode are associated with the frequency of clinical complications and death[184,185]. Increased systemic TNF-α[186] and ascitic Macrophage inflammatory protein (MIP)-1β[187] levels at admission have been found in cirrhotic patients who subsequently developed SBP compared with those of uninfected patients, and also a more severe immune response is detected in cirrhotic patients with SBP compared with non-cirrhotic patients with sepsis[188].

Infected ascitic fluid of decompensated cirrhotic patients displays a decrease of both the CD4/CD8 ratio (due to reduction in CD4 and maintenance of CD8) and T cell receptor (TCR) γδ expression, while the levels of TNF-α were increased compared with non-infected ascites. In turn, ascitic levels of IL-8, IL-10, IL-12 and TNF-α were higher in infected patients defined by ascitic positive bacterial culture[189]. Intracytoplasmic levels of IL-4, the major defining Th2 cytokine, were no different between infected and non-infected groups. These data suggested the predominant cytotoxic response, especially Th1, in cases of decompensated liver cirrhosis with ascitic infections, and the possible role of TNF-α in the pathogenesis of ascites infections[189]. Furthermore, levels of soluble receptors of TNF (sTNFR) in the ascitic fluid were found to be elevated in patients with SBP. The levels of TNF-α, IL-6, IL-1β, C-reactive protein, and the anti-inflammatory molecules, soluble IL-1 receptor antagonist (sIL-1Ra), sTNFR55 were elevated in patients with SBP compared to cirrhotic controls[190,191]. SBP was also associated with significantly higher chemokines (IL-8, Gro-α and MCP-1) soluble adhesion molecule ICAM-1, which could be implicated in the peritoneal infiltrate in patients with SBP[192].

As prognosis markers of bacterial infections and more concretely of the Syndrome of systemic inflammatory response in cirrhosis, besides the clinical indexes MELD and Child-Pugh, the presence of bacterial DNA[193], LPS binding protein and C reactive protein[194], IL-22[195], HLA-DR's expression and IL-10[164,196], among others have been proposed.

DIFFERENCES BETWEEN THE PERITONEAL INFLAMMATORY STATUS IN CIRRHOTIC ASCITES INDUCED BY ALCOHOL AND HCV INFECTION

Kiyici *et al*[189], also segregated cirrhotic patients by the viral or non-viral cirrhotic etiology, showing that the levels of TNF-α were higher in the infected group than in non-infected only in viral etiology; IL-8 was higher in the infected group than in non-infected only in non-viral cirrhosis; and no differences were detected on IL-12 and IL-10 cytokine levels between infected or non-infected group respect to the etiology. Related to this, our group has reported that cirrhosis promoted by HCV is associated with a prevalent immune inhibitory status in both ascites and isolated peritoneal M-DM, contrasting with the cirrhosis induced by alcohol[197]. Ascites associated with HCV-C contains a significant higher number of leukocytes (T lymphocyte, polymorphonuclear and monocyte cell subpopulations) than ALC-C. These findings did not agree with the cellular distributions recorded in the peripheral blood. This indicates that the absolute cell number and population distribution of peritoneal leukocytes in ascites from cirrhotic patients vary with the underlying cause and do not match the situation in peripheral blood. Moreover, the findings also reveal that leukocyte migration towards the peritoneal cavity is not a passive process induced by hemodynamic alterations associated to cirrhosis, like portal hypertension, but is more the consequence of an active chemoattractant-induced process, recruiting leukocytes not only from the blood, but also probably, from impaired lymphatic drainage. Hence, a specific differential profile of chemoattractant stimuli must be implicated in the recruitment of each particular cell population, depending on the cirrhotic etiology. These results[197] also revealed that ascites from HCV-C patients displays a significant lower concentration of IL-12 and a higher level of IL-10 than the corresponding from the ALC-C group, indicating a predominant Th2/Treg pattern in the pathogenesis of advanced HCV-C. However, when cytokine levels of ascitic fluid were compared with the number of macrophages contained there, or secreted *in vitro* by isolated peritoneal macrophages, besides discrepancies in IL-12, the concentration of IL-6 and TNF-α were also different between both groups of cirrhotic patients. Moreover, this fact was related with a lower baseline ERK1/2 phosphorylation in HCV-C than that detected in the ALC-C group. Peritoneal macrophages are "differently primed" by the *in vivo* pathophysiological environment and maintain their inflammatory differentiation pattern for at least 24 h, being more pro-inflammatory under ALC-C condition. This "alert state" could be useful for preventing the development of SBP in recurrent events of intestinal BT in ALC-C patients. Peritoneal macrophages showed a predominant immune inhibited status in the last-stages of HCV-induced liver damage compared with ALC-C[60], which may be produced to avoid immune-mediated decompensation. The drop of significant differences in the IL-10 levels with respect to the number of peritoneal macrophages, clearly suggests that other cell populations are contributing to the total amount of this anti-inflammatory cytokine in ascites of the HCV-C group, essentially Treg cells. However, this does not imply that these cells in HCV-C are functionally exhausted or endotoxin-tolerant as described[198], since they are able to be further activated by several PAMP agonists. In fact, the relative increase of IL-6 secretion and ERK1/2 phosphorylation after stimulation with LPS was significantly higher in HCV-C than in ALC-C patients. Similar results were observed for IL-6 secretion induced by oligodeoxynucleotides (ODN) and *Candida albicans,* and IL-10 induced by ODN from HCV-C patients. These findings also indicate the differential expression and/or susceptibility to agonists of PRRs in the macrophages contained in ascites from both groups of cirrhotic patients.

The smaller inflammatory profile of ascites from HCV-C patients compared with those with alcoholic etiology could also be the consequence of the hypothetically lower frequency of intestinal BT in HCV-C patients.

CONCLUSION

This review focuses on new findings about the inflammatory status involved in the development of human hepatic cirrhosis induced by the two major causes, infection with hepatitis C virus or chronic alcohol abuse, avoiding results obtained from animal models. Described differences between the inflammatory status of cirrhosis caused by these etiologies highlight the interest to perform new studies that allow to obtain further insights into the cellular and molecular mechanisms implicated in this disease. Advances in this field will contribute to a better development of individually tailored preventive and therapeutic procedures.

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TOPIC HIGHLIGHT

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Ultrasound-based elastography for the diagnosis of portal hypertension in cirrhotics

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Abstract

Progressive fibrosis is encountered in almost all chronic liver diseases. Its clinical signs are diagnostic in advanced cirrhosis, but compensated liver cirrhosis is harder to diagnose. Liver biopsy is still considered the reference method for staging the severity of fibrosis, but due to its drawbacks (inter and intra-observer variability, sampling errors, unequal distribution of fibrosis in the liver, and risk of complications and even death), non-invasive methods were developed to assess fibrosis (serologic and elastographic). Elastographic methods can be ultrasound-based or magnetic resonance imaging-based. All ultrasound-based elastographic methods are valuable for the early diagnosis of cirrhosis, especially transient elastography (TE) and acoustic radiation force impulse (ARFI) elastography, which have similar sensitivities and specificities, although ARFI has better feasibility. TE is a promising method for predicting portal hypertension in cirrhotic patients, but it cannot replace upper digestive endoscopy. The diagnostic accuracy of using ARFI in the liver to predict portal hypertension in cirrhotic patients is debatable, with controversial results in published studies. The accuracy of ARFI elastography may be significantly increased if spleen stiffness is assessed, either alone or in combination with liver stiffness and other parameters. Two-dimensional shear-wave elastography, the ElastPQ technique and strain elastography all need to be evaluated as predictors of portal hypertension.

Key words: Portal hypertension; Transient elastography; Acoustic radiation force impulse elastography; Two-dimensional shear-wave elastography

Core tip: Ultrasound-based elastographic methods are being used more and more for the non-invasive assessment of liver fibrosis, with very good accuracy in diagnosing cirrhosis. Transient elastography is a

promising method for predicting portal hypertension in cirrhotics, but it cannot replace upper digestive endoscopy. The diagnostic accuracy of employing acoustic radiation force impulse elastography in the liver to predict portal hypertension is debatable. It may be significantly increased if spleen stiffness is assessed, whether alone or in combination with liver stiffness and other parameters. Two-dimensional shear-wave elastography, the ElastPQ technique and strain elastography all need to be evaluated as predictors of portal hypertension.

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INTRODUCTION

Almost all chronic liver diseases can evolve to liver cirrhosis, which is characterized by profound changes in liver structure that are caused by pseudo-nodule formation as a consequence of necrosis and fibrosis. The main causes of chronic liver disease are infections with hepatitis B and C viruses (HBV and HCV), but a rising incidence of alcoholic and non-alcoholic steatohepatitis (ASH and NASH, respectively) has been observed in developed countries[1]. Published data from the World Health Organization (WHO), state that 160 million individuals are infected with HCV, representing 2.35% of the population, with the highest prevalence in Egypt (20%) and the lowest prevalence in northern European countries (< 0.5%)[2]. It is estimated that up to 30% of HCV patients will develop cirrhosis at least 10 years after diagnosis[3]. The prevalence of HBV is even higher, with more than 240 million chronically infected patients; the highest prevalence is reported in sub-Saharan Africa and South-East Asia (5%-10% in adults) and the lowest is reported in Western Europe and North America (< 1%)[4]. It is estimated that 650000 deaths occur each year as a consequence of liver cirrhosis and hepatocellular carcinoma (HCC) secondary to chronic hepatitis B infection[4]. Alcoholic cirrhosis is also a major cause of disease burden and death. In 2010, 493300 deaths were attributable to alcoholic cirrhosis worldwide (7.2 per 100000 people; 47.9% of all liver cirrhosis deaths)[5]. In Europe, also in 2010, 43500 deaths were caused by alcoholic cirrhosis[5]. More than 5500 liver transplants are performed in Europe each year, 59% of them for liver cirrhosis; of these, 33% come from a purely alcoholic etiology, and 5% come from a mixed alcoholic and viral etiology[6].

It is easy to diagnose decompensated liver cirrhosis in which clinical, biologic and ultrasound signs are evident. However, the most important aspect of prognosis is the differentiation between compensated cirrhosis and chronic hepatitis. Until a few years ago, liver biopsy (LB) was considered to be the reference method for staging chronic hepatitis[7]. However, because it has some drawbacks, including sampling variability and intra- and interobserver variability[8-10], and most importantly because it is an invasive method, noninvasive tests were developed to stage chronic hepatitis, ergo, to diagnose cirrhosis. These noninvasive tests are either serologic, including the FibroTest and ActiTest[11], or elastographic.

Elastographic methods evaluate a property that is intrinsic in every tissue: elasticity, which is the capacity of a tissue to deform and then return to its initial shape when an extrinsic force is applied. Regarding the liver, more fibrosis means that the tissue is less elastic (stiffer). Liver stiffness (elasticity) can be evaluated by magnetic resonance elastography[12,13] or by ultrasound-based elastographic methods[14,15]. According to the guidelines and recommendations published by the European Federation of Societies of Ultrasound in Medicine and Biology (EFSUMB) regarding the clinical use of ultrasound elastography, ultrasound-based elastographic methods can be classified as the following: (1) strain elastography (quasi-static, qualitative elastography), which includes real time-elastography (RT-E); and (2) shear-wave elastography (SWE) (quantitative elastography), which includes a: transient elastography (TE); b: Point SWE [acoustic radiation force impulse elastography (ARFI) and the ElastPQ technique]; and c: real-time SWE [including two-dimensional SWE (2D-SWE) and three-dimensional SWE (3D-SWE)][14,16,17].

It is a known fact that patients with advanced cirrhosis have shorter survival rates due to severe complications such as: portal hypertension (development of esophageal varices - EV), HCC, and hepatorenal syndrome. Thus, it would be advantageous to identify the patients who are at risk for these complications and to screen them: for EV by upper endoscopy and for HCC by ultrasound.

Portal hypertension is one of the most feared complications of cirrhosis. It can lead to the development of esophageal and gastric varices (EV and GV, respectively) and upper digestive bleeding due to variceal rupture. The best method to assess bleeding risk is measurement of hepatic venous pressure gradient (HVPG), available only in specialized centers, and an invasive procedure. In clinical practice, the size of EV is used to assess bleeding risk. According to Baveno V and AASLD Consensuses, primary prevention of variceal bleeding should be applied to patients with large (grade 2 or 3) EV[18,19]. To diagnose clinically significant EV (large-grade 2 or 3 EV), a screening program inclusive of periodic upper digestive endoscopy should be implemented. However, repeated endoscopies are often poorly accepted by patients and are also expensive. Thus, it would be very useful to

Table 1 Predictive value of transient elastography in the liver for clinically significant portal hypertension

Study	HPVG ⩾ 10 mmHg				HVPG ⩾ 12 mmHg			
	Cut-off (kPa)	AUROC	Se	Sp	Cut-off (kPa)	AUROC	Se	Sp
Vizzutti *et al*[30], *Hepatology* 2007	13.6	0.990	97%	92%	17.6	0.92	94.0%	81.0%
Bureau *et al*[31], *Aliment Pharmacol Ther* 2008	21	0.945	89.9%	93.2%	-	-	-	-
Reiberger *et al*[34], *Wien Klin Wochenschr* 2012	18	0.871	83.4%	82.2%	20.0	0.79	84.2%	80.7%
Salzl *et al*[35], *Ultraschall Med* 2014	16.8	0.870	89.7%	75%	-	-	-	-
[1]Shi *et al*[46], *Liver Int* 2013	-	0.930	90%	79%	-	-	-	-

[1]Meta-analysis: HSROC and summary Se and summary Sp are presented. HPVG: Hepatic venous pressure gradient; Se: Sensitivity; Sp: Specificity; AUROC: Area under the receiver operating characteristics curve.

find a non-invasive, inexpensive technique to predict the occurrence of significant varices and the risk of bleeding. It was logical to evaluate the proficiencies of various elastographic methods in this regard because they have been proven to be very accurate in predicting the presence of cirrhosis.

TE

TE was the first elastographic method that was developed to evaluate LS as a predictor for fibrosis[20]. It uses a FibroScan device (Echosens, Paris, France) that includes a special ultrasound probe (3.5 MHz for the standard M probe) integrated into a piston that "punches" the body surface. The "punch" generates shear waves that propagate into the liver. Their velocity is measured by pulse-echo ultrasound acquisition and is proportional to LS, increasing in parallel with LS. The FibroScan device displays a Young's modulus, expressed in kilopascals (kPa), which is proportional to the shear-wave velocity[14,16,17]. Measured values range from 2.5 to 75 kPa.

Liver stiffness is measured in the right liver lobe, in fasting patients. The software automatically rejects measurements with an inconsistent vibration shape or follow-up. For a reliable assessment, ten valid shots should be obtained, and the median value of these measurements should be considered indicative of the LS value. The technical quality parameters that should be considered for correct LS measurements include the success rate (SR) and the interquartile range (IQR). SR is the percentage of valid shots from the total number of shots, while IQR is a measure of variability and is calculated as the difference between the 75th and the 25th percentile of obtained values. Thus, TE measurements are regarded as unreliable if 10 valid shots cannot be obtained or if the IQR is higher than 30% of the median value and/or the SR is lesser than 60%. If no valid shots are obtained, TE measurement is considered failed[21].

Several published meta-analyses have demonstrated that LS measurement by TE is a reliable method for diagnosing cirrhosis, with a pooled sensitivity ranging from 84.45% to 87% and a pooled specificity ranging from 91% to 94.69%[13,22]. In the most recent meta-analysis, for a mean optimal cut-off of 15 kPa, the summary sensitivity was 0.83 for diagnosing cirrhosis with summary specificity of 0.89[23]. However, the cut-off values for diagnosing cirrhosis may vary according to its etiology[24]. Published studies have found the following cut-offs: in HCV infection - 12.5 kPa[25], in HBV infection - 13.4 kPa[26], in NAFLD - 10.3 kPa[27], in ASH - 22.4 kPa[28], and in cholestatic chronic diseases - 17.3 kPa (primary sclerosing cholangitis and primary biliary cirrhosis)[29].

Several studies were published regarding the correlation between TE measurements and HVPG (Table 1). The first studies were performed in rather small numbers of patients. In an Italian study on a small number of patients, the AUROC for predicting HVPG ⩾ 10 mmHg was 0.99 with 97% sensitivity, while for predicting HVPG ⩾ 12 mmHg the calculated AUROC was 0.92 with 94% sensitivity. The calculated cut-offs were 13.6 kPa for HVPG ⩾ 10 mmHg and 17.6 kPa for HVPG ⩾ 12 mmHg. The cut-off was also 17.6 kPa for predicting any EV (90% sensitivity, AUROC = 0.76)[30].

In a French study, TE accurately predicted HVPG > 10 mmHg (significant portal hypertension); the AUROC was 0.945. The calculated cut-off was 21 kPa[31]. In another French study by the same group, which followed up for 2 years 100 patients evaluated by TE and HVPG, similar performances of TE and HVPG for predicting portal hypertension were observed, with AUROCs of 0.830 and 0.845, respectively. During the follow-up, none of the patients with LS values lower than the calculated cut-off (21.1 kPa) had complications of portal hypertension, *vs* 47.5% of those with values higher than the cut-off[32].

In an Austrian study including 122 cirrhotics with EV, a stronger correlation was observed between LS measurements by TE and HVPG in patients with HVPG lower than 12 mmHg (r = 0.951) *vs* those with HVPG higher than 12 mmHg (r = 0.538) and that the correlation improved in patients who were hemodynamic responders to beta-blocker therapy (r = 0.864), while in non-responders it remained the same (r = 0.535)[33]. The calculated cut-off to discriminate between patients with grade 1 EV and those with grade 2 or 3 EV was 47.5 kPa, with good sensitivity (80.6%) but rather poor specificity (47.7%)[33].

The same Austrian group evaluated 502 patients by TE and HVPG. They observed a strong correlation between LS assessment by TE and HVPG (r = 0.794;

Table 2 Predictive value of Transient Elastography in the liver for esophageal varices

Study	At least grade 1 EV				At least grade 2 EV			
	Cut-off (kPa)	AUROC	Se	Sp	Cut-off (kPa)	AUROC	Se	Sp
Nguyen-Khac *et al*[39] *Alcohol Clin Exp Res* 2010	-	-	-	-	47.2 (alcoholic)	0.770	84.6%	63.6%
Nguyen-Khac *et al*[39] *Alcohol Clin Exp Res* 2010	-	-	-	-	19.8 (viral)	0.730	88.9%	55.1%
Sporea *et al*[40] *Med Ultrason* 2013	-	-	-	-	32.5 (alcoholic)	0.836	85%	74.6%
Sporea *et al*[40] *Med Ultrason* 2013	-	-	-	-	24.8 (viral)	0.867	81%	80.7%
Castéra *et al*[41] *J Hepatol* 2009	21.5 (HCV)	0.84	76%	78%	30.5 (HCV)	0.870	77%	85%
Sporea *et al*[45] *World J Gastroenterol* 2011	31	0.78	83%	62%	-	-	-	-
[1]Shi *et al*[46] *Liver Int* 2013	-	0.84	87%	53%	-	0.780	0.86%	0.59%

[1]Meta-analysis: HSROC and summary Se and summary Sp are presented. EV: Esophageal varices; Se: Sensitivity; Sp: Specificity; AUROC: Area under the receiver operating characteristics curve.

$P < 0.0001$). The correlation was stronger in cirrhotics with viral etiology ($r = 0.838$) than in those with alcoholic disease ($r = 0.756$). For a cut-off of 18 kPa, TE had an 86% positive predictive value and an 80% negative predictive value in identifying cirrhotics with clinically significant portal hypertension (CSPH: HPVG $\geqslant$ 10 mmHg)[34].

Recently published studies have also demonstrated a good correlation between TE and HVPG. In a study from 2014, the calculated cut-off to predict CSPH was 16.8 kPa, with 89.7% sensitivity and 75% specificity (AUROC = 0.870)[35]. The same group demonstrated that if new reliability criteria for TE were used [very reliable measurements (IQR/M < 0.1), reliable (IQR/M < 0.3, or > 0.3 if TE < 7.1 kPa) and poorly reliable (IQR/M > 0.3, if TE > 7.1 kPa), where M = the median value], the number of patients who could be evaluated increased (83.2% *vs* 71.6%) without affecting the accuracy of identifying those with CSPH [88.9% (AUC = 0.957) *vs* 89.8% (AUC = 0.962) for a cut-off of 16.1 kPa][36].

As mentioned above, HPVG measurement is an invasive procedure and is not available at many centers; therefore, in clinical practice, upper digestive endoscopy is used to diagnose EV as a sign of portal hypertension. The first published studies demonstrated that LS values lower than 19 kPa had a high negative predictive value for significant EV (grade 2 and 3)[37], with cut-offs varying from 27.5 to 47.2 kPa[38,39]. Additionally, for predicting esophageal bleeding, the calculated cut-off was 62.7 kPa[38].

Predictive LS values for grade 2 and 3 EV were assessed according to cirrhosis etiology (Table 2). The cut-offs were higher alcoholic cirrhosis (47.2 kPa, AUROC = 0.77, 63.6% specificity, 84.6% sensitivity, 92.5% negative predictive value and 44% positive predictive value) than in patients with viral etiology (19.8 kPa, AUROC = 0.73, 55.1% specificity, 88.9% sensitivity, 96.4% negative predictive value and 26.7% positive predictive value)[39]. A similar phenomenon was observed in a study from our group that was conducted on almost 700 patients. In our study, the best LS cut-off for significant EV was 32.5 kPa (AUROC = 0.836) in patients with alcoholic cirrhosis compared with 24.8 kPa (AUROC = 0.867) in those with viral cirrhosis[40].

In a study from 2009 on HCV cirrhosis, Castéra *et al*[41] calculated a cut-off of 21.5 kPa to predict the occurrence of EV, but it had only 76% sensitivity and 78% specificity, thus it was concluded that TE cannot be a replacement for upper endoscopy for the diagnosis of EV. In a smaller, more recent study that compared TE with Forns Index, FIB-4 and Lok score for the prediction of grade 2 and 3 EV in patients with HCV cirrhosis, for a 22.4 kPa cut-off, TE showed the highest accuracy (80%) (AUROC = 0.801)[42].

Chen *et al*[43] evaluated 238 patients with HBV cirrhosis by TE and upper digestive endoscopy. In patients with significant cytolysis ($\geqslant$ 5 × upper limit of normal), for a 36.1 kPa cut-off, TE had 100% negative predictive value with 72.7% positive predictive value for predicting grade 2 and 3 EV, with an AUROC of 0.93[43].

In 2012 Castera published a review article that evaluated only small studies (47-211 patients) with contradicting results: cut-offs for predicting significant EV 19.8-48 kPa; with AUROCs 0.73-0.87. Castera *et al*[44] concluded that "diagnostic performances of TE are acceptable for the prediction of clinically significant portal hypertension but far from satisfactory to confidently predict the presence of EV in clinical practice and to screen cirrhotic patients without endoscopy".

This review did not include a Romanian study conducted on 1000 consecutive cirrhotics[45]. For an optimal cut-off of 31 kPa, we calculated a 76.2% negative predictive value for at least grade 2 EV and a positive predictive value of 71.3%. If the cut-off for at least grade 2 EV was chosen so that the positive predictive value was higher than 85% (> 40 kPa), we calculated 77.8% sensitivity, 68.3% specificity, 86% positive predictive value, with 55% negative predictive value. If the cut-off was chosen so that the negative predictive value was close to 90% (17.1 kPa), we calculated the negative predictive value to be 89.3%, with 43.2% positive predictive value, 92.6% sensitivity and 33.5% specificity for at least grade 2 EV[45]. Thus, according to our calculations, patients

with LS values higher than 40 kPa will have significant portal hypertension in at least 8/10 cases and should receive prophylactic beta-blocker treatment without undergoing endoscopy. Considering that for the 40 kPa cut-off the negative predictive value was 54.9%, 5/10 cases will have significant (grade 2 and 3) EV, thus we recommend endoscopic evaluation. For the 17.1 kPa criterion the negative predictive value for significant EV was 89.3% (1 in 10 patients), thus we do not recommend endoscopic assessment[45].

Finally, a method's value is demonstrated by meta-analyses. Regarding TE and portal hypertension, a meta-analysis that included 18 studies with more than 3,500 patients was published in 2013[46]. It showed that TE had a 0.90 summary sensitivity and a 0.79 summary specificity (AUROC = 0.93) for predicting CSPH (HVPG $\geqslant$ 10 mmHg), a 0.87 summary sensitivity and a 0.53 summary specificity (AUROC = 0.84) for predicting the occurrence of any EV, and a 0.86 summary sensitivity and a 0.59 summary specificity (AUROC = 0.78) for predicting significant (grade 2 and 3) EV. The conclusion was that, due to the low specificity of this method, TE cannot replace upper gastrointestinal endoscopy for EV screening[46].

Spleen stiffness (SS) measurement by TE was also assessed as predictor of portal hypertension based on the idea that splenomegaly is one of the clinical signs of cirrhosis. Several studies found a good correlation between SS and LS by TE in patients with cirrhosis and also between SS and the presence of EV[47,48] or HVPG[49].

In a cohort of 200 cirrhotics, SS values higher than 40.8 kPa had 94% sensitivity, 76% specificity, 91% positive predictive value and 84% negative predictive value for the presence of any EV. Also, SS by TE was significantly correlated with HVPG (r = 0.433, P = 0.001). When combining LS (cut-off 27.3 kPa) and SS (cut-off 40.8 kPa), the accuracy of TE in predicting EV was 90%[50].

Because in many cases a maximum value of 75 kPa is obtained when assessing SS in patients with cirrhosis, the idea of using a modified software version for SS assessment by TE was explored. Using this modified software, which is not commercially available, mSS values of up to 150 kPa could be obtained by TE. In a series of 80 HCV cirrhotics, mSS accurately predicted grade 3 EV: the cut-off was 75 kPa, with 100% sensitivity, 69.01% specificity, 100% negative predictive value and 29% positive predictive value, with an AUROC of 0.903[51]. Similar results were obtained in another study in which multivariate analysis demonstrated that mSS was the only independent factor associated with grade 2 and 3 EV. The cut-off was 54 kPa, with 80% sensitivity, 70% specificity, and an AUROC of 0.82[52].

Finally, the EFSUMB guidelines on the use of elastography state that: "TE has some value for predicting the occurrence of complications of liver cirrhosis, portal hypertension, HCC and liver-associated mortality. It cannot replace upper gastrointestinal endoscopy for identifying patients with varices"[15].

ARFI

ARFI is a point shear-wave elastographic technique that measures LS as a predictor of fibrosis. ARFI was first developed by Siemens and was integrated into a standard ultrasound machine (Acuson S2000™, Siemens, Erlangen, Germany); it is also available in newer models. It evaluates LS in a 10/5 mm region of interest (ROI), placed by the operator in a region free of large vessels, while performing B-mode real-time examination. The probe automatically produces a 262 μs, 2.67 MHz acoustic "push" pulse that induces shear-waves into the liver, which are tracked using ultrasound correlation-based methods[53]. Shear-waves speed is quantified using a patented application of ARFI technology [Virtual Touch Tissue Quantification (VTQ)]. The measurement result is displayed on the screen (expressed in m/s), together with the measurement depth.

Scanning is performed with minimal scanning pressure, *via* an intercostal approach on the right liver lobe, approximately where a LB would be performed, in fasting patients in a dorsal decubitus position to avoid cardiac motion. Measurements should be performed 1-2 cm under the capsule[17,54-56]. No recommendations were made by the manufacturer regarding the quality criteria that should be used, but published papers showed a better correlation with histological fibrosis if quality criteria similar to those from TE were used (SR > 60% and especially IQR $\leqslant$ 30%)[17,57,58].

Several studies have proven that ARFI elastography is a reliable technique to predict cirrhosis when compared to LB, with cut-offs ranging from 1.55 to 2 m/s and AUROCs ranging from 0.89 to 0.937[54,59-62]. Additionally, when ARFI elastography was compared to TE with LB as the reference method, it had similar performance to TE in diagnosing cirrhosis[61,62].

Several meta-analyses have confirmed that ARFI is a valuable method for diagnosing cirrhosis, with mean diagnostic accuracies reported as AUROCs of 0.93[63] and 0.91[64], which are comparable to TE[63,65].

Published studies showed controversial results regarding liver assessment by ARFI elastography as a predictor of portal hypertension (Table 3). A study from our group showed no significant differences between mean LS by ARFI in patients with no or grade 1 EV (2.73 ± 0.71 m/s) *vs* those with grade 2 and 3 EV (2.8 ± 0.71 m/s, P = 0.49), nor between those that had a history of variceal bleeding (2.78 ± 0.81) *vs* those with no bleeding history (2.77 ± 0.7 m/s, P = 0.99)[66]. Additionally, another study from Romania showed a rather poor performance of ARFI elastography in predicting large EV, with an AUROC of only 0.596[67], which is a similar result to that from another European study in which the AUROC for predicting large EV was

Table 3 Predictive value of acoustic radiation force impulse elastography in the liver for esophageal varices

Study	At least grade 1 EV				At least grade 2 EV			
	Cut-off (m/s)	AUROC	Se	Sp	Cut-off (m/s)	AUROC	Se	Sp
Bota *et al*[67], *Ann Hepatol* 2012	-	-	-	-	2.25	0.596	93.4%	28.9%
Morishita *et al*[69], *J Gastroenterol* 2014	2.05	0.89	83%	76%	2.39	0.868	81.0%	82.0%

EV: Esophageal varices; Se: Sensitivity; Sp: Specificity; AUROC: Area under the receiver operating characteristics curve.

0.58[68].

Much better results were obtained in Asian studies (Table 3). In a Japanese study, the LS cut-off by ARFI for predicting any EV was 2.05 m/s (83% sensitivity, 76% specificity, AUROC = 0.89), while for high-risk EV the cut-off was 2.39 m/s (81% sensitivity, 82% specificity, AUROC = 0.868)[69].

An European study showed a good correlation (r = 0.646; P < 0.001) between LS measurements by ARFI elastography and HVPG. The calculated cut-off to predict CSPH was 2.58 m/s (71.4% sensitivity, 87.5% specificity, AUROC = 0.855)[35].

Similar to TE, SS assessment by ARFI elastography was evaluated as a predictor of portal hypertension, also with controversial results.

In a study by Rifai *et al*[70], SS performed significantly worse than LS in predicting CSPH (AUROC 0.68 *vs* 0.90), but it must be considered that the LS cut-off calculated to predict CSPH was only 1.67 m/s, which is much lower than that proposed for diagnosing liver cirrhosis[59-62]. The optimal SS cut-off for predicting CSPH was 3.29 m/s, but with rather poor specificity and sensitivity: 73% and 47%, respectively[70]. In a smaller study on only 33 cirrhotic HCV patients, in which only 12 had EV, it was found that evaluating SS by ARFI correlates with the presence of ascites but not with the presence of EV[71].

In a study by Vermehren *et al*[68], the situation was reversed. It was demonstrated by multiple logistic regression analysis that SS by ARFI performed better than LS by ARFI for predicting the presence of large EV, even if the AUROCs of ARFI of the liver and ARFI of the spleen were similar (0.58 for both)[68].

Another study measured liver and SS by ARFI before and after placement of a transjugular intrahepatic portosystemic shunt (TIPS). The mean LS determined by ARFI did not differ before and after TIPS placement, while SS significantly decreased after TIPS placement, at 3.65 ± 0.32 m/s before *vs* 3.27 ± 0.30 m/s after TIPS (P < 0.001)[72].

In a study from our group, we combined several parameters to improve the accuracy of ARFI elastography for predicting grade 2 and 3 EV. LS and SS values by ARFI as well as the presence of ascites were associated with significant EV by univariant analysis. Using multiple regression analysis, the following formula to predict at least grade 2 EV was calculated: Prediction of significant EV (Pred EV_{2-3}) score = -0.572 + 0.041 × LS (m/s) + 0.122 × SS (m/s) + 0.325 × ascites (1-absent, 2-present). The optimal Pred EV(2-3) cut-off for predicting grade 2 and 3 EV was 0.395, with 69.6% accuracy (AUROC = 0.721)[67].

Considering these conflicting results, further studies and meta-analysis are necessary to assess the real value of LS and SS by ARFI elastography as predictors portal hypertension in cirrhotics, either alone or in combination.

Published studies have not explained why TE can predict portal hypertension while ARFI cannot. One explanation may be that there is a wider range for TE above the cut-off for cirrhosis (approximately 12.5-13.5 kPa up to 75 kPa) than for ARFI (approximately 1.8-2 m/s up to 5 m/s).

2D-SWE

2D-SWE is an elastographic method integrated into an Aixplorer™ ultrasound machine (SuperSonic Imagine S.A., Aix-en-Provence, France). To obtain a stiffness measurement, tissue is stimulated by an acoustic "push" pulse generated by the transducer, which generates shear-waves in the tissue. Ultrafast imaging technology allows the acquisition of raw radiofrequency data with a very high frame rate, of up to 5000 frames/s, which enables shear-wave speed estimation by a Doppler-like acquisition over a ROI, which is used for tissue stiffness assessment. Elasticity is displayed as a color-coded image superimposed on a standard, grey-scale B-mode image: red denotes stiffer tissues and blue denotes softer tissues. Additionally, a numerical value is displayed together with the standard deviation of the measured elasticity, either in kPa or in m/s[14,16,17,73,74].

2D-SWE measurements are performed under fasting conditions *via* an intercostal approach using 3-5 acquisitions, after which a mean value is calculated[15]. Starting from the first published studies regarding 2D-SWE, this method has proven to be accurate for diagnosing cirrhosis. In hepatitis C patients, the reported AUROCs were 0.94[75] and 0.98[76]. The cut-off value was 10.4 kPa[76]. In hepatitis B patients, the cut-off for cirrhosis was 10.1 kPa (AUROC = 0.98)[77]. In a recently published study in hepatitis B patients, the AUROC for predicting cirrhosis was 0.945 in the index cohort and 0.967 in the validation cohort for a cut-off of 11.7 kPa[78]. In mixed etiology patients, the cut-off for 2D-SWE in predicting cirrhosis was 11.5 kPa (AUROC = 0.914)[79]. In a very recent study, the AUROC of 2D-SWE in predicting cirrhosis was also very

good at 0.926[80].

Regarding 2D-SWE as a tool to predict portal hypertension, we found only two published studies, both in 2015. In the one performed by Kim *et al*[80], LS measurement by 2D-SWE was used to predict portal hypertension assessed by HVPG measurement. For a cut-off value of 15.2 kPa, 2D-SWE accurately predicted CSPH (HVPG > 10 mmHg), with 85.7% sensitivity and 80% specificity (AUROC = 0.819). In a study by Procopet *et al*[81], a very good accuracy of LS by 2D-SWE was observed when quality technical parameters were taken into consideration (SD/median LS $\leqslant$ 0.10 for depth of measurement < 5.6 cm and SD/median LS > 0.10 for depth of measurement $\geqslant$ 5.6 cm). In this category of patients, the best cut-off to predict CSPH was 15.4 kPa (AUROC = 948, with sensitivity and specificity both higher than 90%).

Further larger studies and meta-analyses are needed to assess 2D-SWE as a predictor of portal hypertension.

Strain elastography

Although strain elastography was the original elastographic method used to assess tissue stiffness and is currently available in most high-end ultrasound machines, its value in assessing liver stiffness as a predictor of fibrosis severity is not yet defined due to unstandardized methodology. Initially, hand compression was used for tissue deformation, but in newer systems heartbeats are used to stress the tissue. Elasticity is displayed as a color-coded image superimposed on a standard, gray-scale, B-mode image: red denotes softer tissues and blue denotes stiffer tissues. In newer systems, a histogram and 11 parameters are also displayed[17]. Recent studies showed promising results when using strain elastography to predict cirrhosis using both elastic ratios[82,83] and average strain histograms[84]. No data are available regarding the use of elastic ratios and average strain histograms obtained by strain elastography for the assessment of portal hypertension.

ElastPQ technique

The ElastPQ technique is the newest elastographic method to appear on the market. It is also a point-SWE technique, which was developed by Philips, and is integrated into the iU22 ultrasound system (Philips Medical Systems, Bothell, WA, United States). An ultrasonic pressure wave generated by the transducer induces shear-waves in liver tissue, whose speed is measured by the Doppler functions of the system within an ROI that is placed by the operator in a desired location using B-mode standard ultrasound. A numeric value indicative of liver stiffness, which is expressed either in m/s or kPa, is displayed on the screen[17]. Promising results following the use of the ElastPQ technique to predict cirrhosis were obtained in both HCV and HBV patients[85-87]. No data are available regarding the predictive value of the ElastPQ technique for portal hypertension.

CONCLUSION

In conclusion, we suggest that all elastographic methods are reliable for the early diagnosis of cirrhosis, especially TE and ARFI, whose value has been proven by meta-analyses. While TE is a promising method to predict portal hypertension in cirrhotics, it cannot replace upper digestive endoscopy. The diagnostic accuracy of LS assessment by ARFI in predicting portal hypertension in cirrhotics is debatable. The accuracy of ARFI elastography may be significantly improved if SS is also assessed, either alone or in combination with liver stiffness and other parameters. 2D-SWE, the ElastPQ technique and strain elastography all need to be evaluated as predictors of portal hypertension.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Genetic, metabolic and environmental factors involved in the development of liver cirrhosis in Mexico

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Abstract

Liver cirrhosis (LC) is a chronic illness caused by inflammatory responses and progressive fibrosis. Globally, the most common causes of chronic liver disease include persistent alcohol abuse, followed by viral hepatitis infections and nonalcoholic fatty liver disease. However, regardless of the etiological factors, the susceptibility and degree of liver damage may be influenced by genetic polymorphisms that are associated with distinct ethnic and cultural backgrounds. Consequently, metabolic genes are influenced by variable environmental lifestyle factors, such as diet, physical inactivity, and emotional stress, which are associated with regional differences among populations. This Topic Highlight will focus on the genetic and environmental factors that may influence the metabolism of alcohol and nutrients in the setting of distinct etiologies of liver disease. The interaction between genes and environment in the current-day admixed population, Mestizo and Native Mexican, will be described. Additionally, genes involved in immune regulation, insulin sensitivity, oxidative stress and extracellular matrix deposition may modulate the degree of severity. In conclusion, LC is a complex disease. The onset, progression, and clinical outcome of LC among the Mexican population are influenced by specific genetic and environmental factors. Among these are an admixed genome with a heterogenic distribution of European, Amerindian and African ancestry; a high score of alcohol consumption; viral infections; a hepatopathogenic diet; and a high prevalence of obesity. The variance in risk factors among populations suggests that intervention strategies directed towards the prevention and management of LC should be tailored according to such population-based features.

Key words: Genomic medicine; Polymorphisms; Viral hepatitis; Obesity; Nonalcoholic steatohepatitis; Risk factors

Core tip: Liver cirrhosis is a global health problem. The onset, progression, and clinical outcome of liver cirrhosis are influenced by several hereditary and lifestyle factors. Worldwide, interactions between genetic and environmental factors involved in liver cirrhosis may be associated with ethnic-based variations. Globally, in populations with admixed genomes, such as the Mexican population, individualized medicine represents a new challenge for hepatologists and gastroenterologists.

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INTRODUCTION

Liver cirrhosis (LC) is a chronic illness caused by multiple, long-term injuries to the liver[1]. Globally, LC represents the 14th leading cause of mortality[2] but is the 12th leading cause of mortality in the United States[1] and the 4th leading cause in Central Europe[3] and Mexico[4]. In general, the main etiologies of LC are chronic alcohol abuse, followed by viral infections (hepatitis C and B viruses) and nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis (NASH)[5]. Other causes that occur in a lesser extent are autoimmune hepatitis, obstructive cholestasis, hereditary hemochromatosis, alpha-1-antitrypsin deficiency, Wilson's disease and drug toxicity[6].

Liver cirrhosis is characterized by the degeneration and necrosis of hepatocytes, the replacement of liver parenchyma with fibrotic tissues and regenerative nodules and loss of liver function[7]. The impaired liver architecture induces intrahepatic vascular distortion and portal hypertension, which manifests in major complications, such as ascites, upper gastrointestinal bleeding, jaundice and hepatic encephalopathy[8]. However, regardless of the etiological factors, the susceptibility and degree of liver damage may be influenced by genetic polymorphisms that are associated with particular ethnic backgrounds[9]. Consequently, genes involved in various metabolic pathways are influenced by distinct environmental lifestyle factors, such as diet, physical inactivity, and emotional stress[10].

Thus, this Topic Highlight will focus on the genetic and environmental factors that may influence the metabolism of alcohol and nutrients in the setting of distinct etiologies of liver disease. Additionally, genes involved in immune regulation, insulin sensitivity, oxidative stress and extracellular matrix deposition may modulate the degree of severity. The interaction between genes and environment in the current-day admixed population, Mestizo and Native Mexican, will be described. The inheritance of a heterogenic distribution of European, Amerindian and African ancestry in this population[11,12] may play a central role in the clinical outcome of liver disease in conjunction with specific environmental factors.

CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN THE PATHOGENESIS OF LIVER CIRRHOSIS

Hepatic fibrogenesis is a key factor for the progression of liver damage as a result of an excessive healing response triggered by acute and chronic liver injury associated with the continuous deposition of extracellular matrix (ECM), mainly fibrillary collagen[13]. The accumulation of ECM is due to both increased synthesis and decreased degradation that contributes to the loss of hepatocyte microvilli and endothelial fenestrations, resulting in LC and liver failure[14,15]. Hepatic fibrosis is driven by a population of activated fibrogenic cells known as myofibroblasts (MFs)[16]. The hepatic stellate cells (HSCs) are the primary source of MFs, orchestrating the deposition of ECM in normal and fibrotic liver, and activation of the immune response[17]. However, recent research has revealed that several cell types contribute to MF formation, including portal fibroblasts and bone marrow-derived mesenchymal cells[18].

The clinical assessment of cirrhosis and the staging of fibrosis have given rise to a number of non-invasive techniques that have been validated as surrogate tests for liver biopsy, such as the most recently developed transient elastography (FibroScan; Echosens, Paris, France). By this method, shear wave velocity correlates with liver stiffness, which is staged from F0 to F4, regardless of the etiological agent of liver fibrosis (Figure 1)[1].

Regarding the cellular and molecular mechanisms of LC, activation of HSCs involves two main stages (Figure 2). The first stage of initiation renders the cells responsive to diverse agents of liver injury, which results from the paracrine stimulation of neighboring cells, such as hepatocytes, circulating leukocytes, platelets and endothelial and Kupffer cells (KCs). Such stimuli include reactive oxygen species (ROS) and inflammatory cytokines. Others effectors are transcriptional factors, such as the three types of peroxisome proliferator-activated receptors (PPAR-α, -β and -γ), c-Myb, nuclear factor kappa B (NF-κB), Kruppel-like factor 6 (KLF6) and the CCAAT/enhancer binding protein-β (C/EBPβ). In addition, growth factors, mainly transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor 1

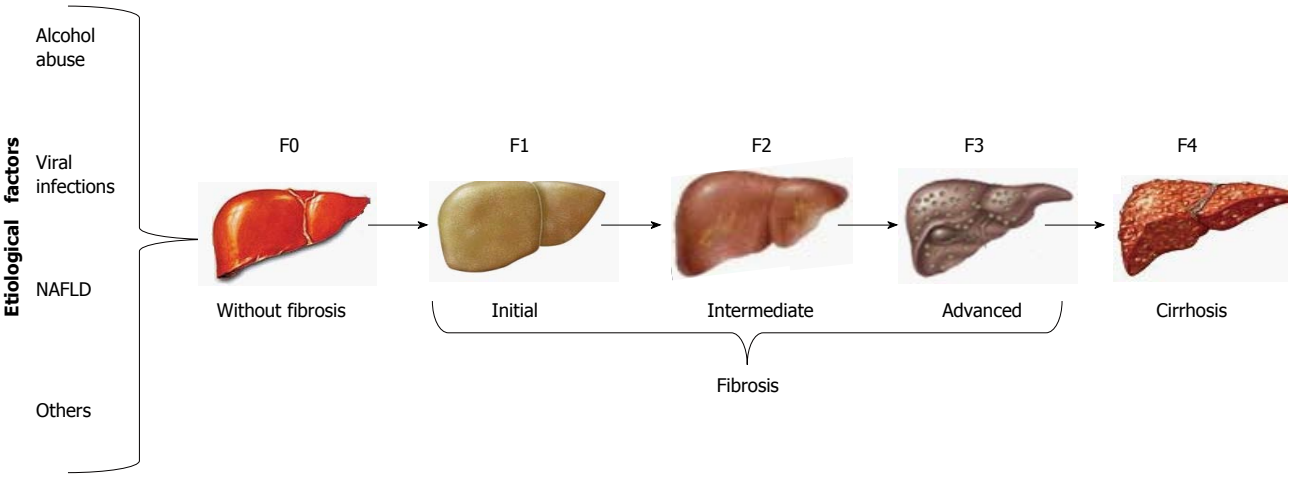

Figure 1 Stages of liver fibrosis. Liver fibrosis may be evaluated by liver biopsy and non-invasive methods. Regardless of etiological factors, liver fibrosis encompasses 3 stages until the development of cirrhosis. NAFLD: Nonalcoholic fatty liver disease.

(IGF-1), play an important role in the activation of HSCs[19,20].

Once the HSCs are activated, the perpetuation stage encompasses phenotypic changes in cell biology, such as proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss, cytokine release and white blood cell chemoattraction. These events are regulated in an autocrine and paracrine manner by proinflammatory, pro-fibrogenic and pro-mitogenic signals to exacerbate the accumulation of ECM[20]. Finally, if the causal agent is eliminated, liver injury may be resolved. In this case, activated HSCs limit the fibrogenic response through the up-regulation of genes involved in apoptosis, enhanced immune surveillance and the increased secretion of ECM-degrading enzymes[21].

GENETIC, METABOLIC AND ENVIRONMENTAL FACTORS INVOLVED IN LIVER DISEASE

Alcoholic liver disease

Overconsumption of alcohol is directly associated with liver disease and affects millions of individuals worldwide[22]. It is estimated that a consumption of > 40 g of alcohol/d in men and > 20 g of alcohol/d in women over a period of 10 years significantly increases the risk of LC[23]. Alcohol-induced liver disease pathogenesis involves alcohol-metabolizing enzymes that alter the levels of acetaldehyde and ROS; these enzymes are known to have variable allelic distribution worldwide (Table 1)[24]. High levels of ROS diminish antioxidant activity, leading to oxidative damage of proteins, lipids, and DNA, which serve as antigens for eliciting the host immune response[25]. In addition, oxidative stress can modify intracellular signaling cascades related to an inflammatory state[26]. The resulting oxidative damage and subsequent mitochondrial and autophagy dysfunction lead to energy depletion, the accumulation of cytotoxic mediators and cell death[27]. Furthermore, acetaldehyde promotes bacterial lipopolysaccharide (LPS) translocation from the gut to the liver, which in turn stimulates Kupffer cells to release proinflammatory cytokines[28]. Additionally, acetaldehyde alters lipid homeostasis by disrupting lipoprotein transportation from the liver, decreasing β-oxidation of fatty acids and increasing its biosynthesis; resulting in hepatic steatosis[29]. Moreover, acetaldehyde is involved in fibrogenesis because this metabolite stimulates the synthesis of collagen and ECM components through the activation of the TGF-β1/SMAD3 signaling pathway[30]. Finally, acetaldehyde and ROS can form DNA adducts that are prone to mutagenesis and carcinogenesis[31].

Furthermore, in alcoholic liver disease (ALD), additional genetic factors involved in alcohol dependence and alcohol abuse have been intensively studied. Among these factors are the dopamine receptor D2 (*DRD2*); the bitter taste receptor (*TAS2R38*); and the liver enzymes alcohol dehydrogenase class Ⅰ, beta polypeptide (*ADH1B*), cytochrome P450, family 2, subfamily E, polypeptide 1 (*CYP2E1*) and aldehyde dehydrogenase 2 family (*ALDH2*). Moreover, polymorphisms of lipid metabolizing genes, such as apolipoprotein E (*APOE*), fatty acid-binding protein 2 (*FABP2*), patatin-like phospholipase domain- containing 3 (*PNPLA3*) and peroxisome proliferator-activated receptor γ2 (*PPAR-γ2*) have been associated with the severity of ALD. Others genes related to the process are inflammatory tumor necrosis factor alpha (*TNF-α*), nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*), CXC chemokine ligand 1 (*CXCL1*) and CD14 endotoxin receptor (*CD14*) (Table 1).

Additionally, environmental cultural factors involved in ALD may vary among populations. In Spain, drinking moderate amounts of red wine is part of the traditional Mediterranean diet and has been associated with reduced all-cause mortality[32]. Mexico has a strong Spanish cultural influence; however, the Mexican

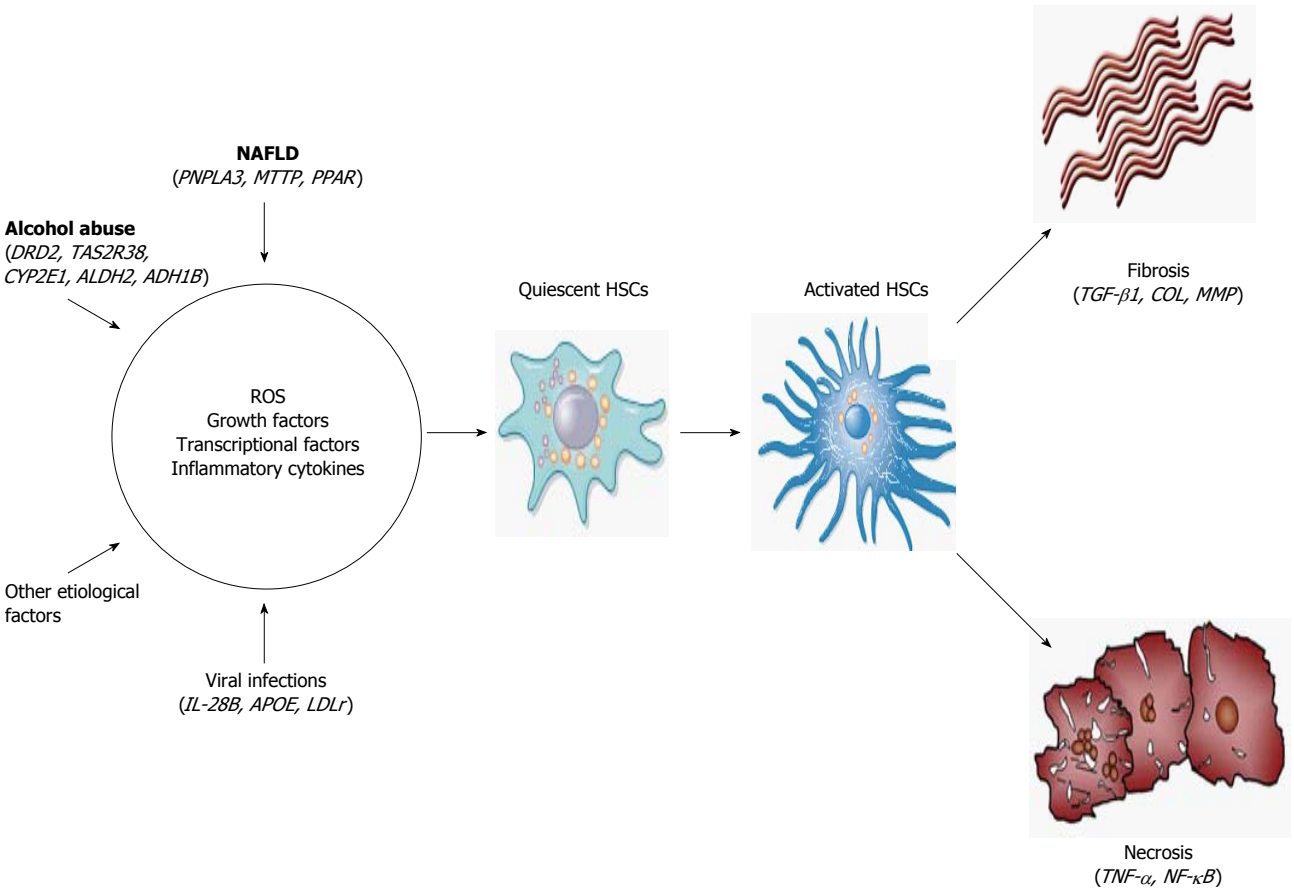

Figure 2 Hepatic fibrogenesis. Different etiological factors induce production of several stimuli to HSCs activation. Activated HSCs promote fibrosis and necrosis of hepatocytes. These pathogenic processes can be modulated by genetic polymorphisms involved in each stage of the pathophysiological process. HSC: Hepatic stellate cells; DRD2: Dopamine receptor D2; TAS2R38: Bitter taste receptor; CYP2E1: Cytochrome P450, family 2, subfamily E, polypeptide 1; ALDH2: Aldehyde dehydrogenase 2 family; ADH1B: Alcohol dehydrogenase class I, beta polypeptide; PNPLA3: Patatin-like phospholipase 3; MTTP: Microsome triglyceride transfer protein; PPAR-γ2: Peroxisome proliferator-activated receptors; IL-28B: Interleukin-28B; APOE: Apolipoprotein E; LDLr: Low-density lipoprotein receptor; TGF-β1: transforming growth factor beta 1; COL: Collagenases; MMP: Matrix metalloproteinase; TNF-α: Tumor necrosis factor alpha; NF-κB: Nuclear factor kappa B; ROS: Reactive oxygen species.

population did not inherit this Mediterranean alcohol drinking pattern. Instead, the Mexican population has a pattern of alcohol consumption that differs from what has been reported in other regions worldwide[24]. Specifically, in West Mexico, young people consume 80 to 360 g of alcohol (mainly beer) over the weekends in a period of 10 years. In the next decade, alcohol consumption eventually increases from 360 to 640 g of alcohol per day, including beer and a combination of beer and distilled beverages, such as tequila. Finally, 3-5 years later, the amount of alcohol increases up to 720 g daily, mainly tequila[24,33], until LC clinically manifests[34,35]. This pattern of alcohol consumption is consistent with the age-related onset of alcohol-induced LC previously reported[36]. The average peak age for LC onset has been reported to be 45 years; however, one group reported an average age of 30 years, which may be the earliest onset ever reported most likely due to genetic susceptibility[36]. Genetic polymorphisms in the *CYP2E1*, *APOE*, and *FABP2* genes have been associated with the early onset of LC in the population of West Mexico (Table 1). However, the distribution of these genetic polymorphisms has demonstrated ethnic differences. Interestingly, the *APOE*2* risk allele is highly frequent in the Caucasian population[12], whereas the *CYP2E1*c2* risk allele prevails among Amerindians[37]. Other factors may affect the development of alcoholic liver damage, including dose, duration and type of alcohol consumption; drinking pattern; gender; obesity; iron overload; and diet[24]. Consistently, patients with chronic alcohol abuse had a higher intake of cholesterol, sodium and simple carbohydrates than other groups with liver disease, which may accelerate hepatocellular injury[38].

Furthermore, certain polymorphisms that are associated with Amerindian ancestry may have placed the current-day Mestizo population at a higher risk of genetic susceptibility to alcoholism. The novel AVV haplotype of *TAS2R38*, which has not been identified in other populations, was associated with alcohol abuse in the population of West Mexico[39]. Globally, Mexico has the highest frequency of the allele A1 in *DRD2*, which has been correlated with alcohol addiction[24]. The identification of these factors has contributed to an integrative understanding of ALD in Mexico. However,

Table 1 Genetic polymorphisms associated with alcohol dependence, alcohol abuse and liver disease

Gene	Risk allele	Association	Population	Ref.
Alcohol-metabolizing enzymes				
CYP2E1	CYP2E1*c2	Higher susceptibility to LC; decompensated liver function	Mexican (Mestizo), West Mexico	[110]
ADH1B	ADH1B*2	Higher risk to LC	Japanese	[111]
ADH1B	ADH1B*1	Alcohol dependence	European, Asian	[112-114]
ALDH2	ALDH2*1	Higher susceptibility to LC	Japanese	[111]
Alcohol dependence genes				
DRD2	Taq I A1	Alcohol dependence	European, East Asian	[115]
TAS2R38	AVV haplotype	Higher alcohol intake	Mexican, (Mestizo), West Mexico	[39]
Lipid metabolism				
APOE	APOE*2	Hypertriglyceridemia and increased development of early LC	Mexican (Mestizo), West Mexico	[36]
FABP2	Ala54	Earlier onset of LC	Mexican (Mestizo), West Mexico	[116]
PNPLA3	M148	Alcoholic liver disease and clinically evident LC	Mixed European and Native American, Mexico City	[117,118]
PPAR-γ2	Ala12	Increased risk to develop severe steatohepatitis and fibrosis	German	[119]
Immune response				
TNF-α	-238 A	Higher prevalence of LC	Spanish	[120]
NF-KB	ATTG deletion	Higher prevalence of LC	Spanish	[121]
CXCL1	rs4074 A	Higher prevalence of LC	German	[122]
CD14	-159 T	Advanced liver disease, hepatitis and especially with LC	Finnish	[123,124]

LC: Liver cirrhosis.

further large-scale studies that involve distinct ethnic groups are needed to determine how genetics may influence the onset and progression of LC.

VIRAL HEPATITIS

Hepatitis C virus

The hepatitis C virus (HCV) is a hepatotropic single-stranded RNA virus of the *Flaviviridae* family[40]. HCV infection is one of the most common causes of chronic liver disease worldwide, with an estimated prevalence of 3% or 170 million infected people[41]. Latin America has one of the lowest prevalences (1.23%); however, this prevalence varies between different regions[42]. In Mexico, 400000 to 1400000 individuals are infected with HCV. Of these individuals, 200000 to 700000 present with active viremia[43]. HCV has been classified into six main genotypes with a heterogeneous global distribution. HCV genotypes 4, 5 and 6 are common in Africa and South East Asia, whereas genotypes 2 and 3 are more frequent in European countries[44]. However, the most predominant HCV genotypes in Mexico are 1a and 1b[45], which have been associated with increased resistance to treatment with pegylated interferon and ribavirin. These HCV endemic differences are explained in part by the prevalent routes of transmission in each region. In West Mexico, the predominant risk factors for HCV transmission are blood transfusions from infectious donors and surgeries with contaminated surgical instruments[46]. In contrast, in North America, injection drug use is the major route of HCV infection[47].

Approximately 18%-34% of patients with acute HCV infection undergo spontaneous clearance through a sustained, vigorous and virus-specific $CD4^+$ T-cell response in peripheral blood[48]. However, in cases of persistent HCV infection, the virus evades the host innate immune response[49]. This condition may potentially increase the risk of progression to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC) over 20-30 years of HCV infection[50]. HCV is a non-cytopathic virus; therefore, the related liver damage is mainly caused by the host immune response. The potential mechanisms of LC include portal lymphoid infiltration, focal and bridging necrosis, and degenerative lobular lesions, which may be amplified by the accumulation of specific T cells that are recruited by adhesion molecules and chemokine expression[51].

Studies in West Mexico have found that cytokines influence the extent of HCV development in patients infected with genotype 1a. Our data reveal that chronic infection results in an increased secretion of interleukin 8 (IL-8) and the chemokine (C-C motif) ligand 5 (CCL5), whereas patients who spontaneously cleared HCV exhibited augmented levels of interleukin 1 alpha (IL-1α), interleukin 13 (IL-13), interleukin 15 (IL-15), TNF-α, TGF-β1 and the chemokine (C-C motif) ligand 8 (CCL8)[52]. This finding correlates with studies conducted in regions where the virus is endemic and suggests that variations in the profile of cytokines involved in the immune response may contribute to either the ability to clear HCV or to the long-term persistence of HCV and thus the potential development of LC[53].

Chronic HCV infection causes numerous pathogenic changes in the liver, including iron overload, steatosis, insulin resistance, the induction of endoplasmic reticulum stress, the unfolded protein response, oxidative stress, mitochondrial dysfunction and altered

growth control[54-56]. Other factors are significantly associated with the progression of fibrosis in chronic HCV carriers. These factors include the duration of infection, advanced age, male sex, heavy alcohol use (> 50 g/d), HIV coinfection, diabetes and a low CD4 count[57-59]. Moreover, physical inactivity, obesity, and related lipid alterations may play a critical role in the progression of fibrosis[42]. Recently, we found that approximately 65% of HCV-infected patients in West Mexico have a sedentary lifestyle, and approximately 70% of these patients are overweight or obese[38]. Because overnutrition may influence the course of infection, the analysis of distinct cohorts, including obese and non-obese individuals, should be conducted to characterize the influence of this factor on LC development.

In addition to environmental and viral features, host genetic factors are important contributors to the modulation of HCV outcomes[42]. Diverse polymorphisms in genes involved in lipid metabolism have been described, such as apolipoprotein B (*APOB*), *APOE*, LDL receptor (*LDLr*), microsomal triglyceride transfer protein (*MTTP*) and *PNPLA3*. Polymorphisms in several immune regulatory genes have been demonstrated to influence HCV outcomes, including *CXCL1*, interleukin 28B (*IL-28B*), *TGF-β1* and *TNF-α*; and matrix metalloproteinase 1 (*MMP-1*), 3 (*MMP-3*) and 9 (*MMP-9*). Additionally, genetic polymorphisms that have been implicated in the metabolism of homocysteine (methylenetetrahydrofolate reductase, *MTHFR*), iron (hemochromatosis gene, *HFE*), and vitamin D (vitamin D receptor, *VDR*) have been reported (Table 2). Finally, a meta-analysis demonstrated that two polymorphisms in *IL-28B* (rs12979860 CC and rs8099917 TT) were strong predictors of sustained virological response in patients on pegylated interferon and ribavirin treatment[60].

Hepatitis B virus

The hepatitis B virus (HBV) is a noncytopathic, hepatotropic virus of the *Hepadnaviridae* family[61]. HBV infection is a public health problem, in which nearly 400 million people suffer chronic HBV infection[62]. In Mexico, epidemiological studies have indicated that at least 15 million adults may have been exposed to HBV during their lifetime[63], and up to 300000 individuals may be active carriers[64]. Globally, geographical areas with a high endemicity of HBV infection include Asian countries, regions in South America with indigenous peoples and Alaska[65,66]. In contrast, Mexico is considered a region of low endemicity[43], which is associated with predominant HBV genotypes H and G[67], low hepatitis B surface antigen (HBsAg) seroprevalence[64], and low viral loads[63] but a high prevalence of occult B infection (OBI) among native Mexican groups (Nahuas and Huichol)[68]. OBI is diagnosed based on a negative serum hepatitis B surface antigen (HBsAg) test, the presence of HBV DNA in the liver and very low HBV DNA levels in serum[69]. Therefore, given these features, the detection of HBV infection in Mexico may be underestimated in the setting of HBV-induced chronic liver disease.

The outcome of HBV infection is the result of complex interactions between HBV and the host immune system[53,70]. A study of cytokine sera profiles in native Mexican groups revealed that OBI patients displayed increased interleukin 2 (IL-2) secretion and a characteristic inflammatory profile (reduction in IL-8 and TNF-α, and increased levels of TGF-β1)[71]. This finding correlates with the accepted role of TGF-β1 in the progression of liver disease[72], whereas the high secretion of IL-2 suggests that OBI may be modulated by IL-2. Experimental data indicate that cytotoxic T cells are the main cell type responsible for the inhibition of viral replication and for hepatocyte lysis[73].

Moreover, HBV genotypes influence the natural course of infection and related liver damage. Regarding the distribution of HBV genotypes, HBV genotype H in native and Mestizo Mexicans and genotype F in native Latin Americans are linked to a less severe natural course of disease and a faster resolution of infection[74]. This finding may be due to a high degree of immune adaptation to the virus in these populations. Nonetheless, in Mestizo Americans, genotype F is commonly associated with acute and chronic liver disease with a tendency toward HCC[74].

Additionally, HBV is associated with metabolic alterations in host cells, which lead to hepatic steatosis, oxidative stress, inflammation, and carcinogenesis. The main mechanisms include the transcriptional up-regulation of genes involved in the biosynthesis of lipids, phosphatidylcholine, and hexosamine; as well as modifications in cell proliferation and differentiation. Together, such changes promote HBV replication and liver damage[75]. Other factors, such as alcohol abuse (> 60 g/d) for at least 10 years, central obesity, insulin resistance, diabetes and environmental hepatotoxins, including tobacco smoke and aflatoxins, may increase the progression rate of liver injury in chronic HBV-infected patients[76]. In addition, genetic polymorphisms modulate the outcome of HBV infection. These polymorphisms include interleukin 10 (*IL-10*), *IL-28B*, *TGF-β1* and the collagenases, type I, alpha 1 (*COL1A1*) and type Ⅲ, alpha 1 (*COL3A1*) (Table 3).

Other viral hepatitis

Recently, we described a high seroprevalence of the hepatitis E virus (HEV) in serum samples from cirrhotic patients in whom no etiological agent was found[43]. Consequently, this finding suggests a potential role of HEV in LC development and is consistent with the description of HEV-related cirrhosis in immunocompromised patients[77]. Further epidemiological studies are warranted among the Mexican population to elucidate the plausible influence of HEV in liver disease.

Table 2 Genetic polymorphisms associated with the outcomes of hepatitis C virus infection

Gene	Risk allele	Association	Population	Ref.
Lipid metabolism				
APOB	-516 C	Increased susceptibility of HCV infection	Chinese	[125]
APOE	APOE*3	Viral persistence	Northern European	[126]
LDLr	rs2738459 C, rs2569540 G, rs1433099 A, rs11672123 A	Higher viral load in genotypes 1 and 4	Spanish	[127]
MTTP	-493 T	Higher degree of steatosis, HCV RNA serum levels and hepatic fibrosis	Native Italian	[128,129]
PNPLA3	M148	Higher risk for steatosis and fibrosis progression	European: Belgian, German and French	[130]
Inmune response mediators				
CXCL1	rs4074 A	Higher risk for LC	German	[131]
IL-28B	rs12979860 T	Higher risk for LC and HCC	Native Italian and Chinese	[132,133]
TGF-β1	-509 T	Higher risk for LC and HCC	Egyptian	[134]
TNF-α	-308 A	Higher risk for LC and HCC	Egyptian	[134]
Fibrogenesis				
MMP-1	-1607 2G	Higher prevalence of LC	Japanese	[135]
MMP-3	-1171 5A	Lower age at LC diagnosis and a higher Child-Pugh score	Japanese	[135]
MMP-9	-1562 C	Higher prevalence of LC	Japanese	[135]
Nutrient metabolism				
MTHFR	677 T	Hyperhomocysteinemia and higher degree of steatosis and fibrosis	Italian	[136]
HFE	63 D	Higher likelihood of LC	Taiwanese	[137]
VDR	CAA haplotype (rs1544410 C, rs7975232 A, rs731236 A)	Higher fibrosis progression and LC	Swiss	[138]

LC: Liver cirrhosis; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 3 Genetic polymorphisms associated with the outcomes of hepatitis B virus infection

Gene	Risk allele	Association	Population	Ref.
Immune response mediators				
IL-10	-592 C	Significant increased risk of LC	Asian	[139]
IL-28B	rs12979860 C	Increased risk for developing LC	Asian	[140]
TGF-β1	10 T	Higher prevalence of LC	Chinese	[141]
TGF-β1	-509 C	Higher susceptibility to LC	Chinese	[142]
Fibrogenesis				
COL1A1	TC haplotype (-1997 T, -1363 C)	Higher prevalence of LC	Chinese	[143]
COL3A1	rs3106796 A	Higher prevalence of chronic hepatitis, LC and HCC	Koreans	[144]

LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

NAFLD

Obesity is considered the main factor associated with multiple metabolic disorders, such as NAFLD, which comprises simple steatosis, NASH, cirrhosis and HCC[78,79]. Hepatic steatosis is defined by the presence of cytoplasmic triglyceride (TG) droplets in more than 5% of hepatocytes as a result of an imbalance between lipid input and output[80,81]. The abnormal accumulation of TGs in the liver induces lipotoxicity, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and inflammation, which all result in fibrosis (Figure 3)[82,83]. Conventionally, international guidelines and intervention strategies have been proposed for the treatment of NAFLD/NASH. However, because disparities in genetic and environmental factors among populations were not considered, the application of these strategies may not be feasible for all populations worldwide. Therefore, intervention strategies for the prevention and management of such obesity-related diseases should be based on a regionalized genome-based diet by focusing on the specific ancestry of each population and the convenience of consuming traditional ethnic food[11].

Currently, Mexico is one of the countries with the highest number of obese individuals, which is closely related to dietary factors and physical inactivity[84]. The diet in West Mexico is hepatopathogenic because of its high content of fat derived mainly from the habitual consumption of meat, fried foods and sausages[11,38]. Interestingly, we have observed that a genetic variant in the fat taste *CD36* receptor may play an important role in this condition[85]. High-fat diets are associated with an increase in body weight, the upregulation of blood glucose levels, the progression of steatosis, and marked inflammation of the liver[86]. Moreover,

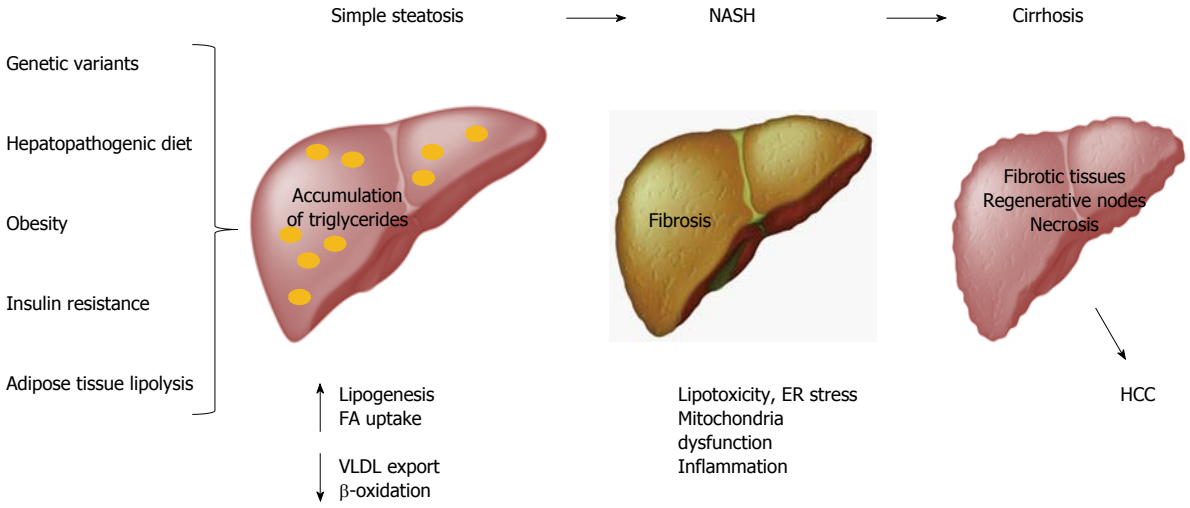

Figure 3 Nonalcoholic fatty liver disease pathogenesis. The spectrum of NAFLD includes simple steatosis, NASH, LC and even HCC. Risk factors such as obesity, hepatopathogenic diet, insulin resistance and adipose tissue lipolysis lead to accumulation of triglycerides. This abnormality can stimulate lipotoxicity, ER stress, mitochondria dysfunction and inflammation, promoting fibrosis. Chronic fibrogenesis causes histological changes in the liver that lead to LC, which in turn may evolve to HCC. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; FA: Fatty acids; VLDL: Very low-density lipoprotein; ER: Endoplasmic reticulum.

more than 70% of the Mexican population frequently consumes soda, which increases fructose input[38]. Fructose promotes steatosis, insulin resistance, necroinflammation, and fibrosis[87-89]. In contrast, our diet is deficient in n-3 polyunsaturated fatty acids (PUFA) and antioxidants, which favors oxidative stress and lipid alterations[90]. Finally, inadequate dietary behaviors, such as missing breakfast and a lack of feeding schedules, are common among the Mexican population[38] and have been linked to obesity[91].

Other interactions between genes and environmental lifestyle factors that favor changes in the body mass index have been studied in West Mexico. Dietary modifications consisting of a lower intake of saturated fat (< 7%) significantly decreased body mass index, waist circumference, waist-to-hip ratio, and reactive C protein in *FABP2* Thr^{54} allele carriers compared to Ala54 allele carriers[92]. Additionally, recent studies in an obese cohort demonstrated that the expression of adiponectin levels may be modulated by changes in lifestyle regardless of the presence of an adiponectin (*ADIPOQ)* 11391G/A polymorphism[93].

However, despite the strong correlation between obesity and NAFLD, approximately 5% of morbidly obese patients do not develop NAFLD[94]. This finding supports the role of genetic factors in the development of NAFLD and NASH (Table 4). These factors may include polymorphisms in genes that affect lipid metabolism, such as apolipoprotein C3 (*APOC3*), *PNPLA3*, *MTTP,* and phosphatidylethanolamine N-methyltransferase (*PEMT*). Polymorphisms in genes that affect insulin sensitivity, such as *ADIPOQ*, adiponectin receptors 1 and 2 (*ADIPOR 1* and *ADIPOR 2*), *PPAR-*γ, PPAR-γ coactivator 1α gene (*PPARGC1A*) and *PPAR-*α, may play a role in the development of NAFLD and NASH. Additionally, regulatory genes of oxidative stress, including glutamate cysteine ligase (*GCLC*), inducible nitric oxide synthase (*NOS2*), and manganese superoxide dismutase (*SOD2*), have been implicated. Genes related to immune regulation, including signal transducer and activator of transcription 3 (*STAT3*), *TNF-*α*, IL-8* and interleukin-6 (*IL-6*); as well as *MTHFR* and *HFE* have been described. Finally, other less common genes that have been associated with the development of NAFLD and NASH include ATP-binding cassette subfamily C member 2 (*ABCC2*, also known as multidrug resistance protein 2, *MRP2*) and angiotensin Ⅱ type Ⅰ receptor (*AGTR1*)[95].

HCC

Globally, HCC is the most common form of liver cancer, the sixth most prevalent cancer and the third most frequent cause of cancer-related death[96]. Nearly 75% of HCC cases are attributed to chronic HBV and HCV infections in high endemic regions[97]. Therefore, the particular characteristics of these viruses and the genetic structure and environmental factors that prevail in each population may explain the epidemiological differences in HCC worldwide. Regions with higher incidence rates of this malignant disease include sub-Saharan Africa, Eastern Asia, followed by Southern European countries[32]. However, HCC is rare in other geographical areas, such as North and South America, Northern Europe, Oceania, and Mexico[98]. The low incidence of HBV-related HCC among the Mexican population is associated with a low steady HBsAg seroprevalence[64]. Furthermore, because Mexican patients with alcoholic cirrhosis are young, death occurs earlier due to long-term complications. This fact hinders the study of the association between alcohol consumption and HCC in

Table 4 Genetic polymorphisms associated with the development of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis

Gene	Risk allele	Association	Population	Ref.
Lipid metabolism				
APOC3	482 T, 455 C	Higher fasting plasma triglyceride concentration and higher prevalence of NAFLD	Asian Indian	[145]
PNPLA3	M148	Increased hepatic fat levels, hepatic inflammation and fibrosis in NAFLD and NASH patients	Hispanic, African American, European American, Finnish, Argentinean, Italian	[146-150]
MTTP	-493 G	Higher intrahepatic triglycerides content. Higher incidence and progression of NASH	French, Japanese	[151,152]
PEMT	M175	Higher prevalence of NAFLD and NASH	Hispanic, African American, European American, Asian	[153,154]
Insulin resistance/sensitivity				
ADIPOQ	45 T, 276 T	Higher prevalence of NAFLD. Lower postprandial adiponectin and higher postprandial triglyceride, VLDL, and FFA in NASH patients	Italian	[155]
	45 G, 276 G	Higher prevalence of NAFLD, severe fibrosis and insulin resistance in females	Japanese	[156]
ADIPOR1	-8503 A, -1927 C	Lower insulin sensitivity and higher liver fat	German	[157]
ADIPOR2	rs767870 T	Increased hepatic fat and biochemical surrogates of NAFLD	Finnish	[158]
PPAR-γ	161 T	Higher susceptibility of NAFLD	Chinese	[159]
PPARGC1A	rs2290602 T	Higher occurrence of NAFLD	Japanese	[160]
PPAR-α	Val227	Higher prevalence of NAFLD and anthropometrical indicators of obesity	Chinese	[161]
Oxidative stress				
GCLC	-129 T	Higher prevalence of NASH	Brazilian	[162]
NOS2	rs1060822 T	Higher fibrosis index in NAFLD patients	Japanese	[163]
SOD2	1183 T	Higher prevalence of NASH	Japanese	[152]
Immune response mediators				
STAT3	rs6503695 T, rs9891119 A	Higher prevalence of NAFLD	Argentinean	[164]
TNF-α	-238 A	Higher prevalence of NAFLD and NASH	Italian, Chinese	[165,166]
IL-8	-251 A	Disease progression in NASH	Turkish	[167]
IL-6	-174 C	Higher risk for NAFLD and NASH	Italian	[168]
MTHFR	1298 C, 677 C	Higher prevalence of NASH	Turkish	[169]
HFE	282 Y	More hepatic fibrosis in NASH patients. Higher prevalence of NAFLD	Australian	[170-172]
ABCC2/MRP2	rs17222723 T, rs8187710 A	NAFLD disease severity	Argentinean	[173]
AGTR1	rs3772633 G, rs3772627 C, rs3772622 A	Higher prevalence of NASH	Japanese	[174]

NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

Mexico because the average age of death from HCC is at least one or two decades after the clinical diagnosis of LC[98]. Similarly, epidemiological studies have indicated a positive correlation between liver cancer and the consumption of several foodstuffs contaminated with aflatoxins and fumonisins, including maize, cereals, ground nuts and tree nuts[99]. These mycotoxins are potent liver carcinogens through the formation of pro-mutagenic DNA adducts. Nonetheless, the process of "nixtamalization" in the elaboration of Mexican tortillas since pre-Hispanic times[100] inactivates up to 95% of aflatoxins in corn (maize), which may exert a protective effect against the development of HCC[101].

EPIGENETIC FACTORS

Epigenetic changes affect gene expression without altering the underlying DNA sequence[102]. These changes include DNA methylation, histone modifications, chromatin remodeling, and microRNAs (miRs), which are essential for the proper maintenance of cellular homeostasis[103]. However, such processes are regulated by environmental factors, including diet, alcohol, drugs, exercise and stress[104]. Growing evidence suggests that epigenetic alterations are correlated with a wide range of chronic disorders, including liver diseases. Diverse miRs have been implicated in the development and progression of NAFLD by disrupting lipid and glucose metabolism, insulin resistance, the unfolded protein response, mitochondrial damage, ER stress, oxidative stress, cellular differentiation, inflammation and apoptosis[105,106]. Moreover, the reduction of histone expression promotes the differentiation of HSCs to myofibroblasts, thereby enhancing the fibrogenesis process[107]. Interestingly, alcohol directly stimulates changes in chromatin structure, which induces the trans-differentiation of HSCs and the increased expression of ECM proteins[108]. Global DNA hypo-methylation and the dysregulated expression of non-coding RNAs and epigenetic regulatory genes have

been found to trigger carcinogenesis of hepatocytes[109]. Taken together, these findings support the need to evaluate epigenetic factors in distinct populations and their relationship to the severity of liver damage.

CONCLUSION

LC is a complex disease. The onset, progression, and clinical outcome of LC are influenced by genetic polymorphisms that are associated with particular ethnic backgrounds and their interaction with environmental factors, such as lifestyle. Currently, chronic alcohol abuse is the main etiological factor of LC in Mexico, which is associated mainly with cultural and genetic aspects. To date, HCV genotypes 1a and 1b favor chronicity, liver damage and therapeutic failure among Mexicans. In contrast, the more favorable natural course of HBV infection in the native Mexican population may be linked to an evolutionary immune adaptation to HBV genotype H. Finally, the hepatopathogenic diet and the high prevalence of obesity in our population contribute to the onset and progression of NAFLD. These findings highlight the influence of genetic and environmental factors in the development of LC, which may be different than those reported in other regions of the world. Therefore, these factors represent a challenge for hepatologists and gastroenterologists in Mexico and worldwide because they require the incorporation of personalized genomic medicine into current medical practices. However, the development of intervention strategies according to individual or population-based features will have a substantial impact on the prevention, management and treatment of LC.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Non-invasive diagnosis of liver fibrosis and cirrhosis

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Abstract

The evaluation and follow up of liver fibrosis and cirrhosis have been traditionally performed by liver biopsy. However, during the last 20 years, it has become evident that this "gold-standard" is imperfect; even according to its proponents, it is only "the best" among available methods. Attempts at uncovering non-invasive diagnostic tools have yielded multiple scores, formulae, and imaging modalities. All are better tolerated, safer, more acceptable to the patient, and can be repeated essentially as often as required. Most are much less expensive than liver biopsy. Consequently, their use is growing, and in some countries the number of biopsies performed, at least for routine evaluation of hepatitis B and C, has declined sharply. However, the accuracy and diagnostic value of most, if not all, of these methods remains controversial. In this review for the practicing physician, we analyze established and novel biomarkers and physical techniques. We may be witnessing in recent years the beginning of the end of the first phase for the development of non-invasive markers. Early evidence suggests that they might be at least as good as liver biopsy. Novel experimental markers and imaging techniques could produce a dramatic change in diagnosis in the near future.

Key words: Liver; Fibrosis; Cirrhosis; Non-invasive; Serum biomarkers; Ultrasonography; Computerized tomography; Magnetic resonance imaging

Core tip: Liver fibrosis (leading to liver cirrhosis), and not inflammation and cytolysis, is the main cause of liver disease-associated morbidity and mortality. During the last 20 years, it has become evident, even to its proponents that liver biopsy, is no longer the "gold-standard". At most, it is the old standard. Non-invasive diagnostic scores, formulae, and imaging modalities, all of which can be repeated as often as required, are cheaper, better tolerated, safer, and more acceptable

to the patient than liver biopsy. Although their accuracy is still controversial, early evidence indicates that they might be at least as good as liver biopsy.

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INTRODUCTION

Until the mid-20th century, chronic liver diseases could be diagnosed ante mortem with certainty only at a very advanced stage, usually after the onset of cirrhosis[1,2]. The first quantifiable noninvasive markers of liver disease were serum levels of liver enzymes. Alkaline phosphatase and transaminases became available in 1930 and 1955-1956, respectively. The spectrum of chronic liver disease expanded, and the submerged part of the chronic liver disease "iceberg" became known. Almost simultaneously (1958) Menghini introduced his "one second liver biopsy" technique and needle. Examination of liver tissue "Intra Vitam" became possible and contributed to the exposure of additional hidden parts of the iceberg, both qualitatively and quantitatively. Various imaging techniques came later and contributed their share.

The introduction of more and more efficient therapeutics in the 1980's transformed hepatology from a mainly descriptive discipline into an active one, able to cure many patients. More precise quantitation of the degree of liver damage became necessary for "To treat or not to treat" decisions, but neither liver biopsy (LB)[3] nor single parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), or platelet numbers were adequate. Standing on the shoulders of giants, Child and Turcotte[4] and Maddrey *et al*[5], investigators combined the power of single parameters by inserting them into various scores and formulae, thus greatly improving their predictive power.

The following review is a practical guide for the clinician.

OVERVIEW OF LIVER FIBROSIS

Liver fibrosis, leading to liver cirrhosis, is the result of several processes, which include the stimulation of fibrogenesis [extracellular matrix (ECM) synthesis] and regulation of fibrolysis (ECM degradation)[6,7]. It is initiated by a variety of insults leading to the death of hepatocytes, prominently viral infections [hepatitis B virus (HBV), hepatitis C virus (HCV)], alcohol, and diet [non-alcoholic fatty liver disease (NAFLD)]. This leads to activation of hepatic stellate cells (HSCs), which is the main mechanism leading to liver fibrosis[8]. HSCs are a main storage for retinol, a precursor of vitamin A, and control ECM turnover by secretion of matrix metalloproteinases (MMPs) and MMP-inhibitors (TIMPs). Three stages are involved in the fibrogenic process through HSC activation: a pre-inflammatory phase of HSC activation by dying hepatocytes, an inflammatory phase, when HSCs are further stimulated to transdifferentiate to myofibroblasts (MFB)[9], and a postinflammatory phase, when MFBs secrete stimulating cytokines and ECM components. These cytokines can stimulate MFBs and HSCs, creating a positive feedback loop that perpetuates the fibrogenic process. The main cytokine mediating this effect is transforming growth factor beta (TGF-β)[10]. TGF-β stimulates ECM gene expression and decreases ECM degradation by downregulation of MMPs and upregulation of TIMPs. HSCs can also be activated through oxidative stress in the form of reactive oxygen species, an important pathway in alcoholic liver injury, NSFLD, and iron overload. The oxidative species can also be produced by activated Kupffer cells. MFBs change the structure of the ECM by altering the types of deposited collagen, laminin, glycoproteins, and proteoglycans (for example heparan sulfate). Changes in the secretion and degradation of ECM components are used as biomarkers for some of the noninvasive screening techniques. The change in ECM structure, in turn, increases ECM stiffness, a change that is measured in some of the physical techniques for noninvasive diagnosis of liver fibrosis.

SERUM MARKERS

Many of the serum markers are enzymes that are measured in routine laboratory tests but are not specific to the liver and can be released upon inflammation of other tissues.

Others are secreted molecules, such as bilirubin, alpha-fetoprotein, alpha-2-macroglobulin, haptoglobin and apolipoprotein A1.

Albumin is specifically secreted from the liver, and its levels are reduced mainly in severe liver disease but also in other clinically relevant diseases (inflammatory diseases, renal diseases with significant proteinuria, malnutrition, protein losing enteropathy). Therefore, although albumin is a good indicator of ill health, it lacks specificity for liver disease.

None of these markers is of much use by itself but are useful when combined in marker panels[11,12].

Combinations of biomarkers or marker panels have been established in recent years for clinical use. The most common ones are summarized in Table 1. They are all based on indirect biomarkers (see below), except for hyaluronic acid or hyaluronan (HA) and panels that include it (Fibrometer and Hepascore).

Although some of these markers, or combinations of them, are now established in clinical use, their prognostic value is not clear-cut. They are increasingly useful in the exclusion of advanced fibrosis and

Table 1 Diagnostic accuracy of established serum markers

Test	Parameters	Prognosis	Sensitivity	Specificity	AUROC
APRI	AST, platelet count	Significant fibrosis	81	55	0.77
		Cirrhosis	77	75	0.84
FIB-4	Platelet count, AST, ALT, age	Significant fibrosis	64	68	0.74
		Cirrhosis	90	58	0.87
Fibrotest	Haptoglobin, α2-macroglobulin, apolipoprotein A1, γGT, bilirubin	Significant fibrosis	92	38	0.79
		Cirrhosis	83	76	0.86
Forns Index	Age, platelet count, γGT, cholesterol	Significant fibrosis	88	52	0.76
		Cirrhosis	98	27	0.87
HA	hyaluronan	Significant fibrosis	-	-	0.75
		Cirrhosis[1]	65	86	0.92
HepaScore	Bilirubin, γGT, hyaluronan, α2-macroglobulin, age, gender	Significant fibrosis	66	79	0.79
		Cirrhosis	72	86	0.89
Fibrometer	Platelet count, prothrombin index, AST, α2-macro-globulin, hyaluronan, urea, age	Significant fibrosis	69	81	0.82
		Cirrhosis[1]	62	87	0.90

[1]All values are medians. Except for these values, which were taken from Ref. [11], all other values are from Ref. [42]. APRI: AST to platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase, γGT: γ-glutamyltransferase.

cirrhosis but do not distinguish well early and intermediate stages of fibrosis, a problem shared with the past "gold standard" - LB[3,13].

However, some novel experimental markers hold promise of improving this noninvasive diagnostic ability in the near future.

The biomarkers can be divided into direct and indirect markers. Direct biomarkers reflect the changes in the ECM structure, including markers of ECM turnover, fibrogenesis, and fibrolysis. Indirect biomarkers are related to liver damage and/or decline in liver function, during the development of fibrosis and cirrhosis. They have also been called class I (direct) and class Ⅱ (indirect) biomarkers[14]. For the sake of clarity, we will discuss established and experimental markers separately.

Established serum markers

AST/ALT ratio: ALT and AST commonly misnamed "Liver function tests" are actually "Liver damage tests", as they are released from damaged cells. Taken together, they yield much more information than each one alone.

De Ritis *et al*[15] proposed the AST/ALT ratio in 1957, only 2 years after these tests were described. Williams and Hoofnagle from the National Institutes of Health (NIH) described very similar findings in 1988: "In the majority of cases of chronic viral hepatitis, the AST/ALT ratio was less than 1.0. However, there was a statistically significant correlation between the AST/ALT ratio and the presence of cirrhosis. Among 100 patients with chronic type B hepatitis, the mean AST/ALT ratio was 0.59 in those without cirrhosis and 1.02 in those with cirrhosis. Furthermore, the AST/ALT ratio often rose to greater than 1.0 when cirrhosis first became manifest. Thus, the finding of an AST/ALT ratio of greater than 1.0 in a patient with nonalcoholic liver disease should suggest the presence of cirrhosis. In addition, the use of the AST/ALT ratio as a means of separating alcoholic and nonalcoholic liver disease must be tempered with the knowledge that this ratio may be less helpful in the presence of cirrhosis".

Testa's group from Genoa showed in 1999 that an AST/ALT ratio of < 1 correctly classified 170 patients suffering from chronic hepatitis, and misclassified seven patients suffering from cirrhosis as suffering from chronic hepatitis. Thus, a ratio < 1 rules out cirrhosis with a great degree of certainty. The AST/ALT ratio performed less well among 171 cirrhotics; indeed, 130 had a ratio > 1, but 41 had a ratio of < 1. There was also a strong correlation between the De Ritis index and monoethylglycinexylidide (MEGX) formation, and indocyanine green (ICG) clearance[16].

It is fascinating to note that 16 years later, in the European Association for the Study of the Liver (EASL) 2015 postgraduate course, one of the take home messages was identical: "Simple and complex serum based tests have > 90% predictive value for excluding cirrhosis, though are poorly predictive of cirrhosis"[17].

McPherson *et al*[18], from Newcastle upon Tyne found that the De Ritis index could avoid LB in 69% of NAFLD patients and had a negative predictive value (NPV) to exclude advanced fibrosis of 95% at a cutoff of 0.8. The other scores, the Bard, the Fibrosis 4 (OFIB-4), and NAFLD fibrosis score, also performed very, well saving 38%-62% of biopsies.

APRI: stands for AST-Platelet Ratio Index. It is calculated in the following way: APRI = [AST level (/ULN)/Platelet counts (10^9/L)] × 100 and is one of the simplest marker panels that can diagnose significant fibrosis and cirrhosis with acceptable accuracy[19]. It has been extensively evaluated in HCV. A meta-analysis including 40 studies and a total of 8739 HCV patients showed that APRI had an area under the receiver operating characteristic (AUROC) of 0.77 for

the diagnosis of significant fibrosis (≥ F2), 0.80 for severe fibrosis (≥ F3), and 0.83 for cirrhosis[20]. Similar results for cirrhosis were found for a group of chronic HBV patients[21]. Recent studies indicate that APRI was comparable to other, more complex established panels in excluding advanced but not moderate fibrosis[22,23]. In a comparison of four tests (FibroTest, APRI, FIB-4, and Forns' Score) before and after telaprevir treatment of 1208 chronic HCV patients, APRI showed the most significant decrease[24], confirming the validity of this test found in previous studies[25,26]. A meta-analysis of 22 studies (n = 4266) showed that the summary AUROCs of APRI for significant fibrosis and cirrhosis were 0.76 [95% confidence interval (CI), 0.74-0.79] and 0.82 (95%CI: 0.79-0.86), respectively. For significant fibrosis, an APRI threshold of 0.5 was 81% sensitive and 50% specific. At a 40% prevalence of significant fibrosis, this threshold had a NPV of 80% and could reduce the necessity for liver biopsies by 35%. For cirrhosis, a threshold of 1.0 was 76% sensitive and 71% specific. At a 15% cirrhosis prevalence, the NPV of this threshold was 91%[27].

The World Health Organization(WHO) guidelines on the assessment of the degree of liver fibrosis and cirrhosis in hepatitis C patients suggested that "In resource-limited settings, the aminotransferase/platelet ratio index (APRI) or FIB-4 tests be used for the assessment of hepatic fibrosis rather than other noninvasive tests that require more resources such as elastography or Fibrotest". (of note, this was a conditional recommendation, based on low quality of evidence)[28].

NAFLD, firmly established as a clinical entity only in 1979[29], is rapidly becoming the most prevalent liver disease in affluent society. Because it is asymptomatic and lacks serological markers, its onset and course are even more insidious than viral and autoimmune liver diseases. Thus, an ultrasound (US) scan of the liver is the first diagnostic step. However, as shown by Tapper's group from Boston on 358 patients with biopsy proven NAFLD, 17.6% of patients diagnosed with steatosis also suffer from "biopsy proven" advanced fibrosis, and 16.7% (one in six) of the patients without US detected steatosis had advanced nonalcoholic steatohepatits (NASH), defined as an NFALD activity score (NAS) score > 4. Clearly, US alone does not suffice, and the authors recommended adding APRI. An APRI value > 1 was the most significant predictor of advanced fibrosis in the study population. The predictors of having advanced NASH are being female, having a body mass index (BMI) of > 30, and an AST > 40. In indeterminate cases, a LB should be seriously considered[30].

In 2015, Xiao *et al*[31] from Chengdu compared APRI and FIB-4, the two most validated noninvasive indices, in a meta-analysis of 39 articles with 9377 hepatitis B patients. For the diagnosis of cirrhosis, APRI had an AUROC of 0.726, and FIB-4 had an AUROC of 0.844. The authors concluded that APRI and FIB4 can identify HBV related fibrosis with moderate sensitivity and accuracy.

FIB-4: Is a combination of four simple variables: AST, ALT, age, and platelet count. It is calculated with the following formula:

The FIB-4 index = [age (years) × AST (IU/L)]/[platelet count (10^9/L) × ALT (IU/L)]$^{1/2}$.

It was initially evaluated in human immunodeficiency virus (HIV)/HCV coinfected patients[32]. FIB-4 performed similarly to FibroTest in the diagnosis of advanced fibrosis and cirrhosis in HCV patients[33], and also in a more recent study of 89 HBV and HCV patients[34]. It was also comparable to APRI, with AUROCs around 0.8[35], and of 0.73 in a recent study of 388 patients[23].

Fibrotest: "Fibrosure in the US" patented by Biopredictive, Paris, France is probably the most validated of the established panels. It is a proprietary combination of five serum biochemical markers (alpha-2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glut amyltranspeptidase, and bilirubin) that are altered with liver fibrosis[36]. Its score is correlated with the degree of liver damage. A meta-analysis of eight studies, including 1842 patients, showed a median AUROC of 0.84 for the diagnosis of advanced fibrosis[37], confirming previous studies that indicated the validity of the test for the diagnosis of advanced fibrosis and cirrhosis but not of mild or intermediate fibrosis[38]. Scores may be influenced by acute inflammation, which leads to increases in serum α 2-macroglobulin and haptoglobin levels[39]. Reduction in the Fibrotest score was also observed after treatment of patients[25,40], although in a recent study the change was not as significant with Fibrotest than with other tests[24].

The Forns index combines four simple variables: platelet count, cholesterol levels, age, and gamma glutamyltransferase (GGT)[41]. In a recent review of 22 studies, the median AUROC obtained for significant fibrosis for the Forns index was 0.76 for significant fibrosis and 0.87 for cirrhosis, similar to that obtained with APRI[42]. The score was also reduced significantly during antiviral treatment[24,25].

Hyaluronan is a high molecular weight glycosaminoglycan that is found in the ECM. It enters the circulation during ECM turnover and is rapidly taken up and degraded in the liver through hepatic endothelial cells. Elevated HA levels may reflect increased production of HA, or reduced clearance of circulating HA and, therefore, may correlate with inflammatory activity and fibrosis. In chronic HCV patients, the AUROC of HA was 0.79 for cirrhosis[43] but was less satisfactory for less severe fibrosis. The AUROC was 0.72 in a recent study of 89 patients[34].

Hepascore combines HA with several other parameters: bilirubin, GGT, alpha-2 macroglobulin, age, and gender[44]. The AUROC for diagnosis of cirrhosis was

Table 2 Diagnostic accuracy of selected experimental serum markers

Marker	Prognosis	Sensitivity	Specificity	AUROC
PIIINP	Significant fibrosis	74	75	0.72
	Cirrhosis	64	66	0.76
PINP	Significant fibrosis	70	73	-
	Cirrhosis	63	73	-
YKL-40	Significant fibrosis	78	81	0.81
	Cirrhosis	80	71	0.80
TIMP	Significant fibrosis	66	72	0.71
	Cirrhosis	91	65	0.90
sH2a + ALT[1]	Significant fibrosis	65	85	0.79
	Advanced fibrosis and cirrhosis	-	-	0.86

[1]Except for these values, which were taken from Ref. [87], all other values are medians from Ref. [11]. PⅢNP: N-terminal pro-peptide of collagen type Ⅲ; PINP: N-terminal propeptide of collagen type Ⅰ; TIMP: Tissue inhibitor of metalloproteinase; sH2a: Soluble H2a; ALT: Alanine aminotransferase.

high, 0.89, but it was not better than other tests for significant fibrosis[42].

Fibrometer, patented by Echosens (Paris, France) combines glucose, AST, ferritin, platelet, ALT, body weight, and age by a proprietary formula[45]. In a recent review, the median AUROC for Fibrometer was 0.82 for significant fibrosis and 0.91 for cirrhosis[42], better when compared directly with APRI and FibroTest. It also showed improvement during antiviral treatment[25].

Cirrhometer, patented by Echosens combines the same parameters as Fibrometer but with specific coefficients targeted for the diagnosis of cirrhosis and was developed by the same group of investigators from Angers, France. Boursier *et al*[46] from that group published a long term (mean of 9.5 years) follow up of 373 patients, amounting to 3508 person years. FIB-4, APRI, and Fibrometer at baseline were actually better than a Metavir fibrosis score at baseline at predicting serious liver related events. Cirrhometer was the only predictor of liver related death. Combining Fibrometer and Cirrhometer yielded a better index than Metavir fibrosis score, FIB4, APRI, Fibrotest, and Hepascore. This is an important paper because of two reasons: first, it shows that serum markers can be better than biopsy, and second, it does not compare the different parameters at one point in time but follows a group of patients longitudinally and then, at the end of follow up, determines which parameters were better prognosticators. These kinds of long term follow up longitudinal studies will most probably yield better prognosis, because until now most studies compared a non-invasive marker against an imperfect standard. Still, as the authors themselves acknowledge, the Fibrometer and Cirrhometer need to be further evaluated.

It has been proposed that combination of several of the tests mentioned above could reduce the need for biopsy[47]. Non-invasive markers for the staging of liver fibrosis are at the edge of replacing liver histology as the gold standard, at least for hepatitis C[48].

Experimental serum markers

Direct experimental markers: Most of the experimental serum markers proposed for the diagnosis of fibrosis and cirrhosis are direct markers related to ECM metabolism. They can be classified as experimental as they are still not widely accepted clinically. The large increase in collagen synthesis by activated HSCs can be an indicator of the fibrogenic process. Collagen is synthesized as a precursor with propeptide extensions at both the N- and C-terminal ends[49]. Before collagen deposition in the ECM, the propeptides are cleaved by N- and C-terminal proteases. The N-terminal pro-peptide of collagen type Ⅲ (PⅢNP) has been the subject of many studies as a marker of liver fibrosis[50,51]. It was reported to detect cirrhosis with a sensitivity of about 94% and specificity of about 81%[52], although other studies showed lower values (Table 2). In a recent study comparing pediatric and adult HCV patients, a significant correlation with advanced fibrosis was obtained in adults (AUROC 0.894) but not in children[53]. Procollagen type Ⅲ amino terminal peptide (PⅢNP) levels are elevated in hepatitis and correlate with aminotransferase levels, and it is more likely a marker of inflammation than of fibrosis[54,55]. The main problem with PⅢNP, as well as with all other ECM-related markers, is that they are not specific for the liver, and their increase can reflect fibrosis or inflammation in other organs. The N-terminal propeptide of collagen type Ⅰ (PINP) has also been studied for the diagnosis of liver fibrosis, but similar to PⅢNP, it may also relate more to inflammation[56]. Type Ⅳ collagen levels have also been correlated to liver fibrosis[57]. The glycoprotein YKL-40, involved in remodeling of the ECM[58], is expressed in liver tissue, particularly in HSCs. Serum concentrations of YKL-40 correlated with other ECM-related markers, such as PⅢNP and HA. Several studies have shown elevated YKL-40 concentrations in the sera of patients with liver diseases. An AUROC of 0.81 was reported for advanced fibrosis in HCV patients[59]. As with other ECM components, YKL-40 can also originate in tissues other than the liver[60]. Laminin levels have also been evaluated for diagnosis of fibrosis, and in a recent study of 87 patients with chronic HBV, it gave 71.9% sensitivity and 80.0% specificity for significant fibrosis[61].

As mentioned above, some cytokines mediate hepatic fibrogenesis and have been investigated as potential markers of fibrosis. TGF-β stimulates ECM synthesis in HSCs. TGF-β levels correlate with the presence of liver fibrosis in patients with alcoholic liver disease (ALD) and HCV[62], and in a recent study, the AUROC obtained for advanced fibrosis was 0.835[53]. TNF-α was associated with fibrosis in ALD[63] and in chronic HBV patients[64]. Platelet-derived growth factor

(PDGF) has also been proposed as a potential marker for fibrosis progression[65]. Connective tissue growth factor (CTGF) is synthesized by HSCs and hepatocytes and is strongly dependent on TGF-β[66,67] and is also related to the fibrogenic process[66]. Its levels also correlate with fibrosis and are decreased in cirrhosis, when fibrogenesis is finally reduced. In studies of CTGF, it gave an AUROC for cirrhosis and fibrosis of 0.955 and 0.887, respectively[68].

The fibrolytic process in the liver is reflected by the serum levels of MMPs and TIMPs. MMP-1 concentrations decrease, while TIMP-1 levels increase during fibrosis in HCV patients[69]. TIMP-1 and MMP-2 (secreted by activated HSCs) correlate well with cirrhosis but the correlation with fibrosis is less clear[69-71].

Indirect experimental markers: Recently, indirect experimental markers have been described and evaluated. Markers of cell damage and death include CK18, evaluated in a group of 143 alcoholics, which could predict severe fibrosis with an AUROC of 0.84[72]. Release of Golgi protein-73 (GP73) was measured in two studies involving 229 and 296 patients with different types of liver disease, showing an AUROC of 0.9 for cirrhosis but much less significant results for fibrosis[73,74]. In a study including 111 individuals with NAFLD, ferritin levels were measured, giving an AUROC of 0.87 for advanced fibrosis and cirrhosis in combination with the BMI. Indicators of oxidative stress, such as malondialdhyde (MDA) and superoxide dismutase (SOD), were found to correlate with fibrosis in a study involving 150 HCV patients, giving AUROCs of 0.9 and 0.8, respectively, for advanced fibrosis and cirrhosis. Again, as mentioned above, the main drawback of all these markers is the lack of liver specificity, as they can be released from other damaged tissues. Interferon (IFN)-L3 expression was reported to be somewhat more restricted to the liver upon viral infection[75]. Changes in IFN-L3 levels were reported to correlate with the response to HCV. In a recent study of 119 chronic HCV patients, serum IFN-L3 increased with advanced fibrosis[76].

An empiric approach has been used in several studies to find differences in the proteome with the development of fibrosis and cirrhosis. In this way, a series of potential markers was identified, *e.g.,* microfibril-associated protein 4 (MFAP-4), which gave an AUROC of 0.97 for cirrhosis and 0.76 for advanced fibrosis[77]. Other identified possible markers in a study of chronic hepatitis C patients were alpha 2 macroglobulin (A2M)/hemopexin with AUROC 0.80 for the detection of significant fibrosis and 0.92 for advanced fibrosis[78]. Also identified in another study as a potential marker was vitamin D binding protein (VDBP) in addition to the established A2M and apolipoprotein (AI)[79]. Similarly, differences in the glycome of patients were investigated. Analyzing binding of serum glycoproteins to a panel of multiple lectins, 183 chronic HCV patients were tested, giving an AUROC of 0.80 for significant fibrosis; 0.88 for severe fibrosis; and 0.93 for cirrhosis, higher than those obtained in direct comparison with several established markers[80]. In a different glycomic approach, the serum N-glycome of 128 chronic HBV patients was analyzed using DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). Selected peak ratios gave correlation with fibrosis, obtaining AUROC of 0.675, 0.736, and 0.754 in the diagnosis of significant fibrosis, advanced fibrosis, and early cirrhosis, respectively[81]. These empiric -omic approaches have the drawback of the complexity of the analysis.

Finally, a series of experimental markers have been identified that are liver specific, an attribute that holds promise for a more specific diagnosis. In a recent study of 293 HBV patients, serum transferrin levels were lower in advanced fibrosis and cirrhosis (F3, F4) than in mild fibrosis (F1, F2). There was, however, an increase in F1, F2, so the difference between no fibrosis (F0) and F3, F4 was very small[82]. The serum levels of complement C3 and C4 beta chains (synthesized in the liver), analyzed by two dimensional gel electrophoresis were found to decrease in HCV patients with cirrhosis[83]. The hepatocyte levels of the asialoglycoprotein receptor are significantly reduced with fibrosis and cirrhosis[84,85]. A soluble form of this receptor (sH2a) is secreted to the plasma and showed very constant levels in healthy individuals and a significant, 3 fold decrease in cirrhosis[86]. A study in HCV patients yielded an AUROC of 0.72 for advanced fibrosis. In a combination with ALT, the AUROCs were 0.86 for advanced fibrosis and cirrhosis and 0.79 for significant fibrosis[87].

EVALUATION OF LIVER FIBROSIS BY IMAGING METHODS

Magnetic resonance imaging

Magnetic resonance imaging (MRI) is used routinely to assess cirrhosis and its complications. However, detection of less advanced stages of fibrosis is more challenging, and several novel MR imaging techniques were used for this purpose[88].

Established modalities

Conventional MRI: Morphologic changes related to cirrhosis can be evaluated with conventional MRI. Macro-structural changes include surface nodularity, widening of fissures, expansion of the gallbladder fossa, notching of the right lobe, and enlargement of the lateral segments of the left lobe and caudate lobe. Parenchymal changes include fibrotic septa and bridges, regenerative nodules, and siderotic nodules or steatotic nodules. Other notable changes in some of the cases are related to portal hypertension, including splenomegaly, porto-systemic varices, ascites, and bowel wall thickening. Administration of intravenous (iv) contrast material improves the visibility of fibrosis

and cirrhosis-related changes and complications of cirrhosis. Fibrosis has a specific enhancement pattern with peak enhancement at the late phases (venous/equilibrium phases). This distinctive enhancement pattern and the reticular appearance enable the differentiation of it from other vascular lesions related to cirrhosis (*e.g.,* arterio-portal shunts, HCC)[89].

Innovative techniques

MR elastography: Similar to sonographic transient elastography (TE), MR elastography is based on the fact that the velocity and wavelength of the wave propagating in the tissue increases as the stiffness of the medium increases, *e.g.*, the fibrotic liver. Specific software and hardware are required to perform MR elastography. A driver device is placed over the patient's right upper abdomen and generates acoustic pressure waves at 40-120 Hz. These waves create shear waves in the liver. The images depict the propagating mechanical wave and a specific algorithm generates a quantitative stiffness map.

In several studies, MR elastography detected advanced fibrosis and cirrhosis in NAFLD patients and chronic hepatitis B patients. The quantitative assessment correlated significantly with the stage of fibrosis. It also proved to be an efficient tool for differentiating lower and higher grades of cirrhosis.

Compared with other MRI techniques, MR elastography is more sensitive for the assessment of liver fibrosis and cirrhosis compared with morphological features detected with conventional MRI. It has also much higher inter-observer agreement compared with MRI and diffusion weighted images (DWI). As opposed to ultrasonography, MR elastography is not affected by lack of acoustic window, obesity or presence of ascites and it is not operator dependent. Huwart *et al*[90] showed that MR elastography is more accurate than US elastography, APRI, or a combination of both, and its coefficient repeatability was better than US elastography.

In a meta-analysis of 12 studies done by Singh *et al*[91] using LB as a standard, MR elastography was found to be highly accurate for the diagnosis of advanced fibrosis independent age, sex, BMI, inflammation, and etiology of the liver disease.

Limitations of MR elastography are its cost and the fact that it is time consuming. Liver stiffness may be affected also from hepatic iron overload, steatosis, vascular congestion, cholestasis, and portal hypertension. In these cases, the accuracy of MR elastography may be altered[90-100].

T1 mapping of the liver: In this method, T1 relaxation time images are acquired and T1 maps are created using the scanner's software. Haimerl *et al*[101] showed that T1 maps after the administration of liver specific contrast medium (Gd-EOB-DTPA) correlated with the stage of cirrhosis, but no correlation was found between fibrosis and the non-contrast enhanced images. Other studies by Allkemper *et al*[102] and Rauscher *et al*[103] found a correlation between cirrhosis and T1 relaxation times in non-contrast enhanced MR.

A study by Banerjee *et al*[104] used T1 mapping for assessment of fibrosis, 1H MR spectroscopy for quantifying lipid content, and T2* sequence for assessing iron overload. An algorithm created an iron corrected T1 value, removing the effect of elevated iron on the T1 value. MR values were compared to the histology data. The corrected T1 value identified fibrosis with sensitivity of 86% and specificity of 93% and correlated strongly with different stages of fibrosis, except for an overlap between mild and moderate fibrosis. Additionally, 1H MR spectroscopy correlated strongly with hepatic steatosis. Hepatic iron content had a strong negative correlation with T2*. In this study, the data for all three parameters- fibrosis, steatosis and iron content- was acquired in a 23 min scan[101-104].

Experimental techniques

Reticuloendothelial specific contrast agents: Few studies have been performed with reticuloendothelial system-specific contrast agents. Superparamagnetic iron oxide (SPIO) causes signal drop in the hepatocyte containing liver parenchyma, and as a consequence, it increases the conspicuity of the detection of fibrotic tissue that is less affected by this contrast agent.

Other studies investigated the double contrast enhanced MRI technique. This technique combines a gadolinium based contrast agent and SPIO in the same study. The synergistic effect of both contrast agents increases the visibility of the fibrotic tissue and helps in differentiating advanced hepatic fibrosis from mild fibrosis. This technique also enabled the quantification of liver texture and its correlation with the stage of fibrosis. However, these contrast agents are not clinically available anymore[105-109].

Susceptibility-weighted MRI: Susceptibility-weighted imaging is a gradient echo sequence with increased sensitivity to the presence of iron, hemoglobin, and calcifications. Measurement of liver to muscle signal intensity ratio was shown to correlate with liver fibrosis with high inter-observer agreement[110].

Diffusion weighted MRI: DWI sequences assess the ability of protons to diffuse within a tissue. This sequence is being used routinely for oncology purposes. The apparent diffusion coefficient (ADC) map is a calculated map derived from the DWI images and correlates with the proton's diffusion ability. Preliminary studies using various hardware and different sequences have attempted to correlate between the reduced ADC value that appears in fibrosis and the degree of fibrosis, but the results were not consistent. In studies by Razek *et al*[111] and Lewin

et al[112], DWI correlated with fibrosis in children and adults. In another study, DWI correlated with stages of fibrosis with sensitivity of 75%-85% (depends on the stage of the fibrosis) and specificity of 68%-94%. In this study, the ability to identify fibrosis was significantly higher for MR elastography than DWI.

The limitations of DWI in the assessment of fibrosis are due to the fact that diffusion is affected by perfusion changes, hepatic steatosis, presence of iron in the tissue, and inflammatory changes. Moreover, the sequence is sensitive to susceptibility and motion related artifacts; and since the quantitative analysis is based on the images, it is also very limited[89,93,111,112].

Perfusion MRI: Parenchymal changes in fibrosis cause gradual obliteration of intrahepatic vessels and sinusoids and slow the passage of blood within the parenchyma. In addition, in portal hypertension, portal flow to the liver decreases and arterial flow takes place. These kinetic flow changes related to fibrosis and cirrhosis can be assessed with dynamic contrast enhanced MRI. This technique was shown to be reliable in the staging of liver fibrosis in patients with chronic hepatitis.

Limitations of the study are related to the fact that perfusion is affected also by the cardiac status, fasting state, hepatic congestion, inflammation, liver masses, and hepatic portal venous flow, and, therefore, the kinetic changes are not reflecting the fibrosis exclusively. Image analysis is a time consuming process; and image quality is not sufficient for assessment of nodules, resulting in two injections of contrast material during the scan[89,113,114].

MR spectroscopy: Assessments of liver fibrosis using MR spectroscopy achieved non-uniform results in different studies. PDE (phophodiester) can be measured by MR spectroscopy with sensitivity and specificity of 81% and 69%, respectively, for differentiating advanced from mild fibrosis. A study by Godfrey *et al*[96] showed poor correlation between the phosphomonoester:phophodiester (PME:PDE) ratio and the stage of cirrhosis. The limitations of this technique are that it is time consuming and requires special hardware and software[115,116].

Computed tomography

Morphological liver changes, signs of cirrhosis and signs of portal hypertension can be detected by computed tomography (CT, splenomegaly, collateral venous circulation, and enlarged portal vein), but CT is less sensitive for less advanced cirrhosis.

Perfusion CT: Perfusion CT may help differentiate minimal fibrosis from intermediate fibrosis in patients with chronic liver disease. Mean transient time is the most sensitive parameter, but there is still large overlap between the different parameters.

Fibro CT: An experimental processing method of conventional CT scan images, which are analyzed by additional software. Optical analysis of CT images of the liver utilizing this technique detected the stage and distribution of liver fibrosis in patients with chronic hepatitis C[117,118].

OTHER PHYSICAL METHODS

Ultrasonography

Conventional US: US is a widely available and low cost modality that has no ionizing radiation, allowing for repeated examinations. For these reasons, it is often performed as the initial modality for evaluation of patients with suspected diffuse liver disease and for non-invasive diagnosis of liver fibrosis.

US findings that suggest progression of fibrosis in patients with chronic liver disease include altered parenchymal echogenicity with coarsened echotexture and surface nodularity that reflects the presence of regenerative nodules and fibrous septa. As cirrhosis progresses, characteristic hypertrophy of the caudate and lateral segment with volume loss of the right lobe of the liver is observed, while in the advanced phase liver atrophy is complete[119]. These findings may lack high sensitivity and specificity, and liver morphology may be normal in the early stage of cirrhosis.

In a prospective comparative study of 85 patients with histologically assessed liver conditions, fibrosis was reliably detected on US examination with a sensitivity of 57% and a specificity of 88%[120].

Several studies have evaluated the performance of US features. Early studies of US criteria accuracy found that by using a ratio of transverse caudate lobe width to transverse right lobe width, cirrhotic livers could be separated from non-cirrhotic liver with a sensitivity of 84%, a specificity of 100%, and an accuracy of 94%[121]. A later study examined the performance of a 2 (nodularity and portal velocity) or 7 (nodularity, portal velocity, liver size, caudate hypertrophy, echogenicity, portal vein diameter, and spleen size) component score for the diagnosis of cirrhosis. The sensitivity was 82.2% and 78.7%, while specificity was 79.9% and 80.1%, respectively. Liver surface nodularity is considered one of the most sensitive and more reproducible US signs when associated with reduction in portal velocity[122,123].

In a more recent study, three US parameters were investigated, liver surface nodularity, caudate lobe hypertrophy, and pattern of hepatic venous blood flow, and compared to histological findings on LB. Hepatic surface nodularity was shown to be the most direct sign of advanced fibrosis, with reported sensitivity and specificity of 54% and 95%, respectively. The addition of other signs, such as caudate lobe hypertrophy, increased the sensitivity but diminished the specificity of US[124].

Doppler and US can also detect the development

of portal hypertension by measuring portal vein diameter, which should not exceed 13 mm in quiet respiration, velocity of flow, hepatofugal flow, ascites, and splenomegaly.

Contrast enhanced US: Contrast enhanced US (CEUS) is used in the characterization of liver tumors. However, in recent years, it has also been used to evaluate liver fibrosis, because of changes in intra hepatic microcirculation (intrahepatic shunts) that can occur in chronic liver diseases with fibrotic evolution. Several measurements have been performed, the arrival time in hepatic veins (HVAT) or more recently the intrahepatic transit time (ITT), which is defined as the time delay between the arrival of contrast in the portal vein and in the hepatic vein, the latter considered as an improved parameter in several studies. In one study, an arrival time of contrast in the hepatic vein below 17 s had 100% sensitivity and 93% specificity for cirrhosis, the HVAT being significantly shorter in cirrhotic patients than in noncirrhotic individuals (chronic liver disease and controls patients)[125,126].

Although HVAT measurement is simple, it has some limitations, *e.g.,* cases with extrahepatic shunts. Staub *et al*[126] used a cut-off of 13 s for the transit time and made the diagnosis of severe fibrosis with a specificity of 78.57%, a sensitivity of 78.95%, a positive predictive value of 78.33%, an NPV of 83.33%, and a performance accuracy of 78.79%[127,128].

CEUS requires additional expertise and adds cost, and this may limit its availability for the routine detection of cirrhosis

Elastography: In the last two decades new US-based methods have been developed. Fibrosis in the liver, as in other tissues, determines a reduction in elasticity or an increase in stiffness. US elastography that can evaluate the tissue stiffness permits a non-invasive estimation of liver fibrosis[129-131]. There are two types of US elastography, strain elastography (SE), also named real time elastography (Hi-RTE), and shear wave elastography (SWE). SE is a qualitative technique and evaluation of the tissue stiffness is obtained after manual compression. SWE is a technique that provides a quantitative measure of stiffness, expressed in meters per second (the shear wave speed) or in kilopascals (Young's Modulus) after an acoustic/mechanical pulse induced by the machine itself.

Among SWE methods, TE (Fibroscan) is the only non-imaging method, while Acoustic Radiation Force Impulse (ARFI) (Siemens, Erlangen, Germany and Philips) and 2D-Real Time Shear Waves Elastography (2D-SWE) (Aixplorer system, Supersonic Imagine, Aix-en-Provence, France) are both imaging methods implanted in US machines.

Real-time elastography - Hi-RTE or SE: Real-time elastography is integrated in a US machine (Hitachi Medical Systems Europe Holding AG, Zug, Switzerland) and is technically different from SWE methods. Hi-RTE relies on tissue deformation induced by operator pressure. Recently, a new linear probe was used to assess the liver parenchyma while the internal compression produced by the heartbeat was considered to stress the tissue.

Hi-RTE is a qualitative method used to assess liver fibrosis, where stiffness is given in the color scale or the semi-quantitative method based on the ratio strain between two regions of interest (ROI). The first data regarding chronic hepatitis evaluated by RTE was published by Friedrich-Rust. RTE was performed in 79 patients with chronic viral hepatitis and compared with histological score after LB. The diagnostic accuracy was 0.75 for the diagnosis of significant fibrosis (fibrosis stage according to METAVIR score > or = F2), 0.73 for severe fibrosis (F > or = F3), and 0.69 for cirrhosis[131]. Tatsumi performed Hi-RTE in 119 patients with chronic liver disease and compared the results with LB, TE, and serum markers. The levels of liver strain measured by real-time TE correlated well with liver stiffness. Hi-RTE showed a negative correlation with fibrotic stages and TE findings, suggesting that RTE is a better test than TE[132]. A very recent study was conducted by Meng in which real-time tissue elastography (TE) and LB were performed in 166 patients with chronic hepatitis B and compared with TE. They found that real-time TE has diagnostic performance similar to that of TE in the assessment of liver fibrosis[133]. Colombo conducted a study that evaluated 45 patients with chronic liver diseases and 27 normal subjects and compared three elastographic methods: TE, ARFI, and Hi-RTE. The AUROCs for predicting significant fibrosis (F $\geqslant$ 2) for TE, RTE, and ARFI were 0.89, 0.75, and 0.81, respectively (TE was significantly better than RTE, and there was no significant difference between TE and ARFI nor between ARFI and RTE). The AUROCs for predicting liver cirrhosis (F = 4) for TE, RTE, and ARFI were 0.92, 0.85, and 0.93 respectively with no significant difference between the three curves[134].

TE: TE is a novel method, and the first clinical data using this technique was published in 2003.

TE (Fibroscan; Echosens, Paris, France) was the first US-based elastographic method to evaluate elasticity by measuring the velocity of elastic shear waves in parenchyma generated by a mechanical push. An Ultrasonic M mode transducer is placed above the right lobe of the liver through an intercostal space and produces a mechanical vibration that generates elastic shear waves that propagate through the tissue. The propagation is followed by pulse-echo US acquisitions, and velocity of the waves is measured and expressed in kilopascals. The velocity of the waves correlates directly with the elasticity of the tissue. The stiffer the tissue, the faster the shear wave propagates. The examination is performed on a non-fasting patient lying on dorsal decubitus with the arm in maximal abduction, and the measurement is taken in the right

intercostal space. TE is rapid, easy to perform, and well-tolerated by patients with results immediately available. The technique is operator-independent. Liver stiffness is computed as the median of 10 validated measurements in accordance with manufacturer instructions. Measurements with an interquartile range of less than 30% of the median value and a success rate of greater than 60% are considered reliable. Several studies have demonstrated the reproducibility of the method[135,136].

TE was first validated for liver fibrosis evaluation in patients with chronic hepatitis C and later evaluated in other etiologies of chronic diffuse liver diseases[137-141]. All these studies have demonstrated that there is no specific cut-off to discriminate liver fibrosis and that it varies according to the etiology of liver disease. Many studies showed that TE is highly sensitive and can differentiate between the absence and mild fibrosis from significant fibrosis and cirrhosis but is not accurate enough to differentiate among stages of mild fibrosis, especially between F0-1 and F2. Using a cut-off value of 6.6 kPa, Sporea *et al*[142] reached the best discrimination between absence of fibrosis/mild fibrosis ($F < 2$) and the presence of moderate to severe fibrosis ($F \geqslant 2$). In the meta-analysis by Friedrich-Rust *et al*[143], the mean AUROC in HCV patients was 0.84 with a suggested optimal cut-off of 7.6 kPa for detecting significant fibrosis ($F \geqslant 2$), and the mean AUROC was 0.94 with an optimal cut-off of 13 kPa for predicting cirrhosis. A more recent meta-analysis published by Tsochatzis *et al*[140] included 40 studies and patients with diverse etiologies of chronic liver disease (chronic hepatitis B, C, alcohol, and other causes of cirrhosis). Data regarding patients with chronic hepatitis C were extracted from 14 studies, and the summary sensitivity and specificity were 0.78 and 0.80, respectively, for predicting significant fibrosis. Data regarding patients with chronic hepatitis B were extracted from four studies, and the summary sensitivity was 0.84 and the summary specificity was 0.78. In this analysis for predicting liver cirrhosis (F4 on biopsy), the summary sensitivity was 0.83 and the summary specificity was 0.89, and the mean optimal cut-off was 15.0 ± 4.1 kPa (median 14.5 kPa). The summary sensitivity and specificity for predicting significant fibrosis were 0.79 and 0.78, respectively. The mean optimal cut-off was 7.3 ± 1.4 kPa (median 7.2 kPa).

Recently the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) issued guidelines in which values above 6.8-7.6 kPa in chronic viral hepatitis may indicate the presence of significant fibrosis ($F \geqslant 2$) with a high probability, while the range 11.0-13.6 kPa may indicate a cirrhotic stage ($F = 4$)[144]. EASL guidelines indicate that TE can be used to assess liver fibrosis (level of recommendation A2) in patients with chronic hepatitis C[145].

TE can also be used to predict complications of cirrhosis, such as portal hypertension, or can have a role in the post-transplant setting[146-148]. The limitations of TE include the requirement for expensive equipment, and lack of standardized cutoffs for diagnosis of fibrosis stages. Moreover, TE cannot be performed in patients with obesity and ascites because of poor penetration.

In the last few years, other US-based SWE methods have been used, integrated into conventional US equipment, enabling visualization of tissue along with assessment of tissue elasticity.

Supersonic shear wave elastography or 2D SWE: Supersonic shear wave elastography (SSWE) use acoustic radiation force to induce microscopic tissue movements, producing shear wave in the tissue. In SWE methods, in contrast with RTE, deformation force and tissue deformation are known and, for that reason, quantitative estimation of tissue stiffness, expressed as Young's modulus (kilopascal) or shear wave velocity (m/s), can be obtained.

Two-dimensional WE is the only method that can provide real-time measurements of liver stiffness[149]. The technique is available on the Aixplorer® system. The patient is placed in the supine position with the right arm in maximum abduction, and a convex probe is placed in the right intercostal space, using the best acoustic window available for liver evaluation. Acquisition is performed on the right liver lobe, and no movement of the probe is recommended in order to avoid motion artifacts and to allow map stabilization. The patient has to hold breath for 3 to 4 s in the expiration phase to acquire a stable image. The SWE box has to be placed in a homogeneous vessel free area away from the Glisson capsule. The elasticity value is displayed on the image, and color mapping in the box is depicted in real time. For quantitative measurements, a round region of interest is placed inside the SWE box, and minimum stiffness and maximum stiffness expressed in kilopascals are recorded. A measurement is considered valid if the region of interest is filled out with color.

Contrary to TE, the method can be used in patients with ascites. The first clinical study was published by Bavu *et al*[150] who evaluated 133 patients with chronic hepatitis C by means of SWE, TE, and, in a subgroup of patients, LB. The AUROCs for elasticity values assessed by SWE were 0.95 for significant fibrosis, 0.96 for severe fibrosis, and 0.97 for liver cirrhosis. In this study, the AUROCs for SWE were better than those from TE performed in the same session for $F \geqslant 2$, $F \geqslant 3$, and F4. Ferraioli *et al*[151] compared SSWE with TE and LB. The cut-off value was 7.4 kPa for $F \geqslant 2$ (AUROC = 0.91), 8.7 kPa for $F \geqslant 3$ (AUROC = 0.99), and 9.2 kPa for $F = 4$ (AUROC = 0.97). The AUROCs were similar to those in the Bavu study. More recently, Leung conducted a study in a cohort of HBV patients, comparing TE and SWE of the liver and of the spleen. SWE of liver had significantly higher accuracy than TE of liver and SWE of spleen in all fibrosis stages. The AUROCs for 2D SWE of liver, TE of liver, and 2D SWE of

spleen were 0.86, 0.80, and 0.81, respectively, for mild fibrosis (F1 stage); 0.88, 0.78, and 0.82, respectively, for moderate fibrosis (F2 stage); 0.93, 0.83, and 0.83, respectively for severe fibrosis (F3 stage); and 0.98, 0.92, and 0.84, respectively, for cirrhosis (F4 stage). Two-dimensional SWE of the liver was the most reliable parameter to assess and evaluate liver fibrosis[152]. A very recent study was conducted by Zheng *et al*[153] that included 198 patients with chronic liver disease from different etiologies (HCV, HBV, autoimmune hepatitis, PBC, drug induced liver disease) using LB as a reference standard for most of them. They evaluated the individual and combined performances of 2D SWE and conventional US in assessing liver fibrosis and cirrhosis to determine when 2D SWE should be added to routine US. Two-dimensional SWE was significantly superior to conventional US in detecting liver fibrosis, but for diagnosis of decompensated cirrhosis, there was no significant difference between 2D SWE and conventional US.

ARFI: ARFI elastography is performed with a Siemens Acuson S2000TM US system. The same principle is used in a Philips system. ARFI imaging is a US-based elastography method integrated in conventional US machines where a region of interest in the liver is mechanically excited with an acoustic pulse inducing localized tissue displacement, which results in shear wave propagation. In this method, a single measurement over a small FOV is obtained (point quantification SWE). As compared with TE, ARFI elastography can be used also in patients with ascites[154]. Usually, 10 valid measurements are performed, and a median value is calculated (expressed in m/s). Compared with TE, ARFI has similar accuracy but lower rates of measurement failures[155].

ARFI was first used and validated in patients with chronic hepatitis C and subsequently in other etiologies of chronic liver diseases[156]. Sporea *et al*[157] found in a large cohort of patients that LS measurement by means of ARFI is a reliable method for predicting fibrosis severity in HCV patients. Similarly to TE, there is a large overlap of ARFI measurements for fibrosis F0-F2, and only severe fibrosis and cirrhosis can be excluded with great certainty. The overall correlation with histological fibrosis was not significantly different for TE in comparison with ARFI elastography. However, TE was better than ARFI for predicting the presence of liver cirrhosis and fibrosis (F $\geqslant$ 1). A meta-analysis that included 36 studies revealed good accuracy of the ARFI imaging for the staging of F $\geqslant$ 2 and F $\geqslant$ 3 with an AUROC of 0.84, and excellent diagnostic accuracy with an AUROC of 0.93 for F = 4[155]. In a retrospective international multicenter study that included 914 patients with chronic hepatitis C (10 centers, five countries from Europe and Asia), all patients were evaluated by means of LB, ARFI and, in a subgroup of patients, also by TE. A highly significant correlation (r = 0.654) was found between ARFI measurements and fibrosis (P < 0.0001), being significantly higher in European as compared with Asian patients (r = 0.756, P < 0.0001 *vs* r = 0.544, P < 0.0001). The predictive values of ARFI for various stages of fibrosis were: F $\geqslant$ 1 - cut-off > 1.19 m/s (AUROC = 0.779); F $\geqslant$ 2 cut-off > 1.33 m/s (AUROC = 0.792); F $\geqslant$ 3 cut-off >1.43 m/s (AUROC = 0.829); and F = 4 cut-off >1.55 m/s (AUROC = 0.842). The cut-offs for predicting significant fibrosis and cirrhosis were different in European *vs* Asian subjects: 1.21 m/s (AUROC = 0.857) and 1.74 m/s (AUROC = 0.892) in European subjects, and 1.32 m/s (AUROC = 0.736) and 1.55 m/s (AUROC = 0.736) in Asian patients[158].

Thirteen studies including 1163 patients with chronic hepatopathies were included in a recent meta-analysis The sensibility and sensitivity were 0.74 and 0.83, respectively, for the detection of significant fibrosis (F $\geqslant$ 2) using ARFI and 0.78 and 0.84, respectively, using TE. For the diagnosis of cirrhosis, the sensitivity and specificity for ARFI were 0.87 and 0.87, respectively, and for TE were 0.89 and 0.87, respectively, for TE. The median optimal cut-off value of liver stiffness assessed by ARFI for the detection of significant fibrosis and cirrhosis were 1.3 m/s and 1.8 m/s, respectively[159]. One study compared the feasibility of three shear waves elastographic methods. In a cohort of 332 patients, with or without hepatopathies, liver stiffness was evaluated by TE, ARFI, and SWE. Reliable measurements were obtained in a significantly higher percentage by means of ARFI as compared with TE and SWE: 92.1% *vs* 72.2% and 92.1% *vs* 71.3%, respectively. In subjects in whom reliable liver stiffness measurements were obtained by all three elastographic methods, the accuracy was similar for ARFI and SWE for diagnosing significant fibrosis and cirrhosis compared with TE[160].

PRACTICAL INTEGRATIVE POINTS AND CONCLUSIONS

We are of the opinion that free, powerful tools like FIB-4, De Ritis Ratio, and APRI, preferably with inexpensive imaging technologies (as discussed above), but possibly without them, should be the first step in the evaluation of liver fibrosis and cirrhosis. A large part, if not an overwhelming majority of liver biopsies could be avoided.

Some of the experimental serum markers, especially those that are liver-specific, combined with novel imaging and physical techniques could create a nearly biopsy-free scenario in the near future.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Do vasopressin V2 receptor antagonists benefit cirrhotics with refractory ascites?

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Abstract

Hyponatremia is a frequent complication of advanced cirrhosis with ascites associated with increased morbidity and mortality. It is caused by an impairment in the renal capacity to eliminate solute-free water and is considered to be related to persistent secretion of vasopressin despite low serum osmolality. This nonosmotic release of vasopressin is mediated by the autonomic nervous system, which senses the underfilling of arterial vascular component. This reduction of effective arterial blood volume is closely related to the development of ascites. Although the short-time effects of vasopressin V2 receptor antagonists (vaptans) on hyponatremia and ascites have been repeatedly reported, their effects on the long-term management of cirrhotic ascites have not been established yet. Considering that their effects on water diuresis and their safety are limited by severe underfilling state of patients, cautious approaches with adequate monitoring are needed to advanced cirrhosis. Proper indication, adequate doses and new possibility of combination therapy should be explored in the future controlled study. As hyponatremia is frequent obstacle to ascites management, judicious combination with low-dose diuretics may decrease the incidence of refractory ascites. Although vaptans show much promise in the treatment of advanced cirrhosis, the problem of high cost should be solved for the future.

Key words: Liver cirrhosis; Ascites; Hyponatremia; Pathophysiology; V2 receptor antagonist

Core tip: Dilutional hyponatremia is a frequent complication with high morbidity and mortality in advanced liver cirrhosis with ascites. It is attributable to disturbed water excretion related to enhanced vasopressin activity on the background of underfilling state in the splanchnic arterial circulation. V2 receptor antagonist is theoretically promising for the future treatment of hyponatremia and ascites. The present review aimed to summarize the pathophysiological backgrounds of ascites and hyponatremia and to introduce major results of all controlled trials. Although there existed several unsolved problems and

controversies on the topic, discussions from the basic standpoints may light up the dark road.

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INTRODUCTION

Hyponatremia is a frequent complication of advanced cirrhosis related to an impairment in the renal capacity to eliminate solute-free water[1]. It becomes an important clinical problem for decompensated cirrhosis associated with increased morbidity and mortality[1]. The failure to excrete solute-free water attributable to persistent secretion of vasopressin despite low serum osmolality has been considered to underlie the development of this hyponatremia[2]. Since vasopressin V2 receptor antagonists or vaptans have selective aquaretic effect, they may open new possibilities for management of hyponatremia in advanced liver cirrhosis[3].

A basic understanding of the pathophysiologic mechanisms leading to water retention and dilutional hyponatremia is required for rational treatment of decompensated cirrhosis[4]. This review introduces the basic mechanisms of ascites and water retention and the major trials of vaptans in patients with liver cirrhosis. It also discusses the areas of uncertainty for the future clinical use of vaptans.

PATHOGENESIS OF ASCITES IN CIRRHOSIS

The pathogenetic events leading to ascites formation in patients with liver cirrhosis are extremely complex, being associated with multiple factors[5,6]. Pathophysiologic backgrounds and main theories of ascites formation in liver cirrhosis are shown in Figure 1. Cirrhotic patients exhibit the hyperdynamic circulation characterized by arterial hypotension, increased cardiac output, and reduced systemic vascular resistance[7]. These changes come from peripheral vasodilation and arteriovenous shunt. The contraction of the effective plasma volume associated with peripheral vasodilation may induce compensatory responses such as mobilization of vasoactive hormones and activation of the sympathetic nervous system[8].

Renal vasoconstriction is a common finding in patients with cirrhosis and ascites and more intense in the renal cortex, which may lead to a reduction of renal blood flow and glomerular filtration rate (GFR)[9]. Free water clearance and urinary sodium (Na) excretion are markedly decreased in those with tense ascites. Water and Na retention in advanced cirrhosis is due to increased tubular reabsorption. Increased reabsorption in the proximal convoluted tubule leads to a reduced delivery of filtrate. An increased reabsorption in the distal convoluted tubule may further enhance water and Na retention.

Three main hypotheses, the underfilling theory, the overflow theory and the peripheral arterial vasodilation theory, are considered to explain variable pathophysiological changes in a patient with advanced liver cirrhosis. In the underfilling theory, excess lymph accumulation in the peritoneal space attributable to imbalance of Starling forces in the hepatic sinusoids and splanchnic capillaries is considered to bring contraction of circulating plasma volume, which is thought to constitute an afferent signal to the renal tubule to augment salt and water reabsorption[6]. The postsinusoidal venous block and the decreased albumin synthesis in the background of cirrhosis are important initial events. In the overflow theory, renal Na retention and plasma volume expansion are considered to precede rather than follow the formation of ascites[10]. The investigators who supported this hypothesis[5,11,12] speculated that an increase in sinusoidal hydrostatic pressure directly stimulates renal sympathetic nervous system through the hepatorenal reflex[13,14], which initiates primary renal Na retention. Schrier *et al*[15] proposed the peripheral arterial vasodilation theory as a revised underfilling theory. In this theory, peripheral arterial vasodilation is considered to cause imbalance of capacitance and volume, which leads to decreased effective intravascular volume.

A wide variety of neurohumoral derangements may influence renal handling of salt and water[8]. The activities of the two major vasoconstrictor and antinatriuretic systems, the renin-angiotensin-aldosterone (RAA) system and the sympathetic nervous system, are enhanced in most cirrhotics with tense ascites[9]. Increased plasma level of arginine vasopressin (AVP) also known as antidiuretic hormone (ADH) increases water reabsorption in the collecting duct and contributes to water retention[9].

The mechanism of splanchnic arterial vasodilation in cirrhosis is complex and still undetermined[16]. For many years, arterial vasodilation in cirrhosis has been attributable to increased circulating vasodilators such as estrogen, VIP, prostaglandins[6], CGRP[17], substance P[18], adrenomedullin[19], atrial natriuretic factor[20,21] and nitric oxide[16,22]. As the most important site of vasodilation is the splanchnic circulation, increased production of nitric oxide, that acts in a paracrine manner, has recently been considered essential[16]. Bacterial endotoxin closely related to bacterial translocation is known to stimulate these vasodilators especially nitric oxide[23,24].

BACKGROUNDS OF REFRACTORY ASCITES

Refractory ascites was internationally defined as

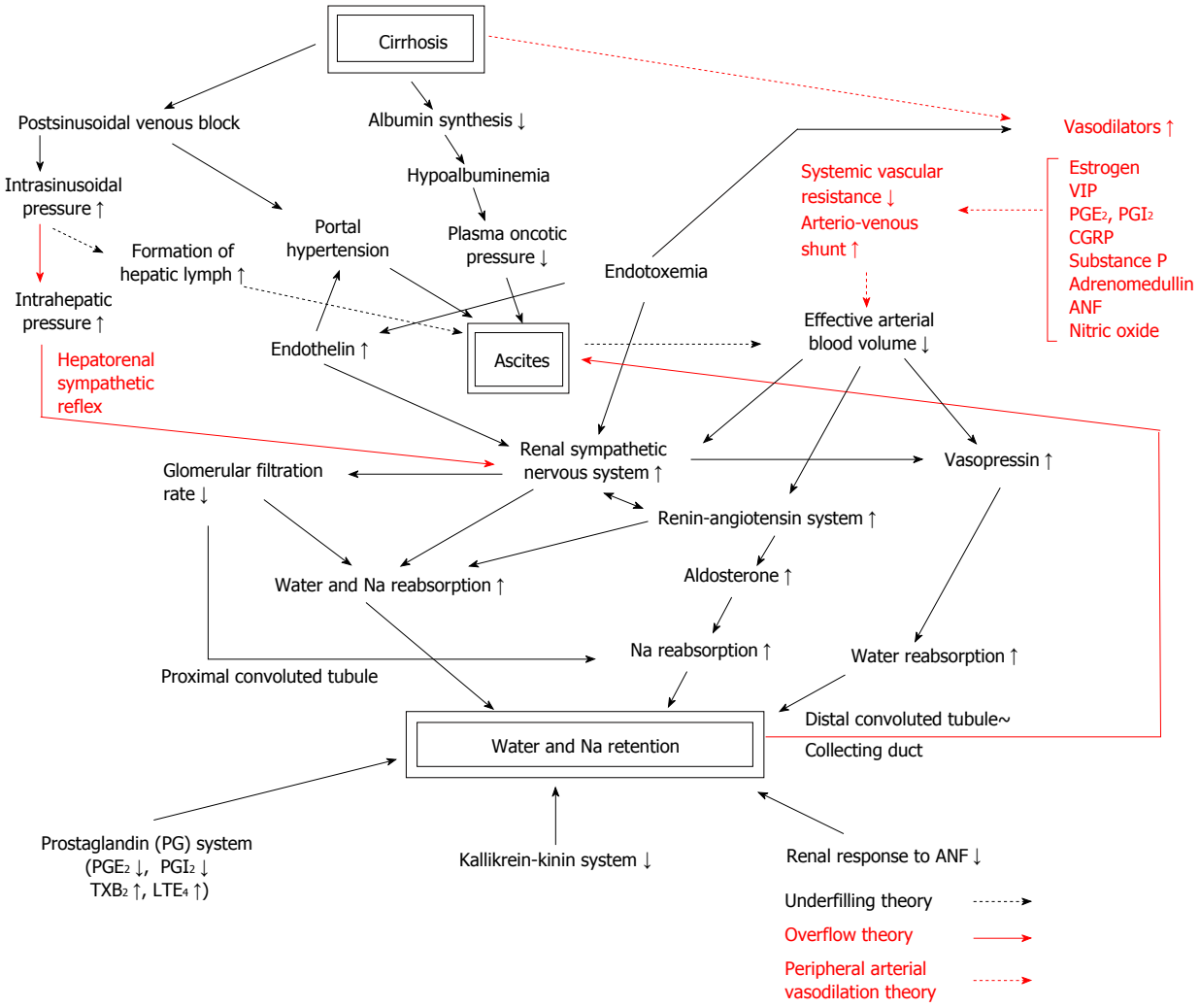

Figure 1 Phatophysiologic backgrounds and main theories of ascites formation in liver cirrhosis. The pathogenetic events leading to ascites formation in patients with liver cirrhosis are multifactorial. They include hepatic venous outflow block, portal hypertension and hypoalbuminemia as hepatic factors, hyperdynamic circulation, peripheral arterial vasodilation, decreased effective circulating blood volume and altered neurohumoral systems as systemic circulatory factors, and enhanced salt and water reabsorption in proximal and distal nephron and convoluted duct related to intrarenal haemodynamic derangement as renal factors. They are closely interrelated and enhance salt and water reabsorption in the kidney. The activities of the two major vasoconstrictor and antinatriuretic systems, the renin-angiotensin-aldosteron system and the sympathetic nervous system, are increased in most cirrhotics with tense ascites. Increased plasma level of arginine vasopressin enhances water reabsorption in the collecting duct and contributes to water retention. Endothelin, an endothelial-derived peptide with marked vasoconstrictor activity, is also increased in advanced cirrhosis[86,87]. Increased endothelin levels were proven to be related to creatinine clearance, effective renal plasma flow[88], serum creatinine and blood pressure[89], and may also contribute to renal dysfunction in patients with cirrhosis[88]. Three main hypotheses, the underfilling theory, the overflow theory and the peripheral arterial vasodilation theory, are considered to explain variable pathophysiological changes occurred in a patient with advanced liver cirrhosis. VIP: Vasoactive intestinal peptide; CGRP: Calcitonin gene-related peptide; PG: Prostaglandin; TX; Thromboxane; LT: Leukotriene.

ascites that does not recede despite Na restriction and maximal diuretic therapy (furosemide 160 mg/d and spironolactone 400 mg/d) or that recurs shortly after therapeutic paracentesis[25]. However, most Japanese cirrhotics with ascites develop electrolyte disturbance or azotemia by this high-dose diuretic treatment. In order to avoid diuretic-induced side effects, we designed a stepped care protocol with a combination of low-dose aldosterone antagonists (400 mg/d of potassium canrenoate, iv) and loop diuretics (40-80 mg/d of furosemide, iv) and studied the pathophysiological backgrounds of these patients[26,27]. In the early step responders, basal renal function, serum Na concentration, plasma renin activity (PRA), plasma levels of aldosterone, norepinephrine (NE), and AVP were within normal limits and basal plasma α-human atrial natriuretic peptide (α-hANP) was elevated[26]. On the contrary, in the late step responders and nonresponders, basal PRA and plasma NE and AVP were progressively elevated with the concomitant decreases in basal creatinine clearance (Ccr), urine volume, urinary Na excretion (UNaV), serum Na levels and the increases in basal blood urea nitrogen (BUN) and serum creatinine levels[26,27]. These results indicate that the early responders were basically in the state of vascular overflow, while the late responders and nonresponders were relatively in the state of vascular underfilling. The pathophysiological backgrounds of

ascites are considered to be a continuum involving both overflow (early stage) and underfilling states (late stage). Interestingly enough, in the patients who responded diuretics, PRA, plasma aldosterone and NE were elevated and α-hANP was lowered by the treatment, suggesting that the diuretics themselves may cause relative vascular underfilling[26].

In the advanced stages of decompensated cirrhosis, patients develop low arterial pressure attributable to further reduction of the peripheral vascular resistances[28]. An increase in the cardiac output to refill the expanded intravascular bed and the release of vasoconstrictors (RAA system, sympathetic nervous system and AVP) to raise peripheral vascular resistance are two compensatory mechanisms to overcome a further reduction of the peripheral vascular resistances and to maintain the hemodynamic stability[28]. Reduced renal perfusion and further Na and water retention with dilutional hyponatremia are the natural consequences of this physiological response[28].

WATER RETENTION IN LIVER CIRRHOSIS

An impaired renal water handling, leading to inability to excrete a water load and hyponatremia, represents a common finding in advanced liver cirrhosis[29]. In refractory ascites, hyponatraemia often develops, which indicates more intense water retention[30]. Free water clearance, an index of water excretion, has been reported to be markedly decreased in patients with cirrhosis and ascites.

On the other hand, the results reported in patients with compensated cirrhosis are still conflicting. By an intravenous water overload of 20 mL/kg body weight, we noted that free water clearance was decreased with the progression of liver cirrhosis[18]. Several other authors[29,31,32] showed a blunt diuretic and natriuretic response to water administration in non-ascitic cirrhotic patients. Conversely, Krag *et al*[30] reported that the Child B cirrhotic patients had increased free water clearance and distal fractional water excretion during a 400 mL/h oral water load.

Pathogenesis of water retention is not fully established, but two major mechanisms have been considered: (1) increased nonosmotic release and decreased clearance of AVP; and (2) decreased GFR and excessive proximal tubular Na reabsorption resulting in impaired free water excretion. For many years, the role of enhanced AVP activity in the cause of subnormal dilution capacity and water retention has received strong support[4]. Taking these results, the current pharmacological therapy has been focused on the release or action of AVP[33]. On the other hand, Gatta *et al*[29] found that free water clearance corrected for distal Na delivery were normal both in non-ascitic patients and in the majority (75%) of decompensated cirrhotics. They thought that excessive proximal Na reabsorption and reduced distal delivery of fluid may play a primary role in the pathogenesis of the impaired water excretion of these patients[29]. Krag *et al*[30] noted that Child C cirrhotics with ascites and mild hyponatremia showed a low GFR, a low distal tubular flow and an inability to increase free water clearance during water loading, while plasma AVP levels remained low. In general, the above two mechanisms are hardly evaluated separately, for the maneuvers leading to expansion of extracellular fluid volume not only enhance the distal delivery of filtrate but may also suppress AVP release by attenuating underfilling of arterial vascular compartment[4].

PLASMA LEVELS OF AVP

After the development of sensitive radioimmunoassay, most investigators reported elevated plasma AVP levels in liver cirrhosis[4]. However, its relation to clinical findings including water excretion was variable. Pérez-Ayuso *et al*[34] reported that plasma AVP levels in cirrhotics were higher than those in normal subjects and plasma AVP levels in cirrhotics with negative free water clearance were even higher than those in cirrhotics with positive free water clearance.

Castellano *et al*[35] found that basal AVP levels were elevated only in decompensated cirrhotics with hyponatremia, although water diuresis and fractional proximal Na excretion were significantly decreased in patients both with and without hyponatremia. They concluded that impaired water excretion in those without hyponatremia cannot be ascribed to enhanced AVP activity but may be related to reduced delivery of filtrate to the distal segment of the nephron[35].

Although basal plasma AVP levels were not different between well-compensated cirrhosis and healthy controls[32,36], suppression of plasma AVP after water load (20 mL/kg body weight) noted in healthy controls was not observed in cirrhotics[36]. Nicholls *et al*[37] evaluated diuretic responses to head-out water immersion, another maneuver to increase central blood volume, and reported that the cirrhotic patients with impaired water excretion revealed higher plasma AVP levels than those with moderate water excretion during water immersion. Bichet *et al*[38] reported that redistribution of central blood volume by water loading in addition to water immersion to the neck resulted in suppression of AVP levels and improvement in water excretion in decompensated cirrhotics. Epstein *et al*[39], however, failed to demonstrate plasma AVP suppression with their water immersion despite increased diuresis in most decompensated cirrhotics. They concluded that the diuresis in some patients without concomitant suppression of plasma AVP suggests that AVP may constitute a permissive rather than pivotal factor in the impaired water excretion in patients with advanced liver disease[39]. The discrepancies among these results on plasma AVP levels may be partly explained by the differences in the basal and post-immersion central blood volumes. The patients in Bichet's study[38], who received a

stronger central hydration (immersion plus intravenous water load) are considered to reveal more effective suppression of AVP[4].

We designed a "body compression" apparatus as a means to restore effective blood volume in cirrhotics with ascites[40]. All four limbs and the lower abdomen were compressed with constant pressure for 3 h, using stroke rehabilitation splints, while patients lay supine[40]. Repeated body compression alleviated ascites in those with well-preserved renal function, but was ineffective in those with markedly impaired renal function[40]. In the responders, plasma AVP levels were within normal limits during the study, whereas in the nonresponders, markedly elevated AVP was not depressed by the body compression[40].

NONOSMOTIC RELEASE OF AVP

The possible mechanism for increased AVP activity in patients with liver cirrhosis may include enhanced nonosmotic release of AVP, decreased hepatic metabolism and enhanced tubular sensitivity to AVP[4]. Among them, enhanced nonosmotic release of AVP is considered to play most pivotal role. In physiological state, mechanisms of osmoregulation and volume regulation help to maintain water balance and tonicity in the body[41]. AVP is synthesized by two hypothalamic nuclei (supraoptic nuclei and paraventricular nuclei) and secreted by the posterior pituitary in response to an increase in plasma tonicity or decrease in plasma volume. Effective circulating arterial volume acts as a nonosmotic factor which regulates the secretion of AVP[41].

The major nonosmotic pathway for AVP release involves the autonomic nervous system, which is mediated *via* the baroreceptors located mainly in the left ventricle and carotid sinus[42]. These baroreceptors communicate to the hypothalamus *via* parasympathetic pathways and cause a release of AVP in response to hypovolemia[33]. Small changes of < 10% in blood pressure or blood volume have no effect on AVP levels. However, once decreases in volume or pressure exceed this value, baroreceptor-mediated signals provide persistent stimuli for AVP secretion[43]. In cirrhotic patients with ascites, the nonosmotic release of AVP from the posterior pituitary becomes the dominant force and the end result is impaired free water excretion and subsequent dilutional hyponatraemia[44].

The peripheral arterial vasodilation of splanchnic vascular bed attributable to vasodilators such as nitric oxide, is considered to cause typical circulatory derangement in liver cirrhosis, *i.e.,* hyperdynamic circulation. Although total plasma volume may be increased in these circumstances, splanchnic arteriolar vasodilation leads to underfilling of arterial vascular component. The reduction of "effective plasma volume" is sensed by the high-pressure osomoreceptors and stimulates RAA system and sympathetic nervous systems, resulting an increase in AVP release[4].

ROLE OF AQUAPORIN-2

AVP plays an important role in water and Na homeostasis. It acts *via* three receptor subtypes-V1a, V1b, and V2-distributed widely throughout the body[41]. V1a receptors are present on vascular smooth muscle cells, myocardium, platelets, and hepatocytes, and mediate vasoconstriction, platelet aggregation, and glycogenolysis[41]. V1b is expressed in the anterior pituitary gland where it mediates adrenocorticotropin release[45]. V2 receptors are located in principal cells of the renal collecting duct system and mediate water reabsorption. AVP acts on V2 receptors, which activate the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway and translocate intracellular vesicles of water channel aquaporin-2 (AQP-2) to the apical plasma membrane of collecting duct cells. This AQP-2 in the cell surface increases the reabsorption of free water from the tubular fluid back into the circulation[1,41,46]. AVP further regulates the long-term water permeability of the collecting ducts by increasing AQP-2 gene expression[46] and AQP-2 protein synthesis[47]. AVP thus plays a pivotal role in the on-off regulation of the cellular trafficking of AQP-2 and the synthesis of AQP-2 in collecting duct cells[47]. Nonsuppressible release of AVP is profoundly involved in abnormal antidiuresis in pathological states of impaired water excretion[47]. Such a chronic AVP excess is closely associated with abundance of AQP-2 protein in collecting duct cells despite hypoosmolar condition[47].

In experimental studies, increased expression of AQP-2 mRNA and protein were also found and closely related to volume of ascites in CCl_4-induced cirrhotic rats[48]. Jonassen *et al*[49] conversely showed that the renal expression of the AQP-2 was down-regulated in bile duct-ligated cirrhotic rats with Na retention but without ascites. They further demonstrated that renal AQP-2 expression in membrane fractions of both whole kidney and inner medulla from CCl_4-induced cirrhotic rats with hyponatremia and ascites was unchanged[49].

In clinical investigations, Ivarsen *et al*[50] showed that urinary AQP-2 excretion was increased in parallel with impairment of free water clearance with the progression of liver cirrhosis, although it was not related to plasma AVP levels. They concluded that there is a functional association between increased AQP-2 excretion and increased renal reabsorption of water in cirrhosis. Chung *et al*[51] also reported that urinary AQP-2 secretion was increased in patients with liver cirrhosis, especially in those with ascites. In contrast to these results, Esteva-Font *et al*[52] found a progressive decrease in urinary AQP-2 excretion as the progression of liver cirrhosis, from compensated cirrhosis to cirrhosis with ascites and hepatorenal syndrome. Plasma AVP level did not correlate with urinary AQP-2 excretion and markedly increased in

those with hepatorenal syndrome[52]. Krag *et al*[30] reported that plasma AVP level was suppressed but AQP-2 excretion was unchanged and urine volume, free water clearance and distal fractional water excretion were not increased after water load in Child C patients. These discrepancies between plasma AVP level and urinary AQP-2 excretion support an uncoupling of AVP/AQP-2 system attributable to a AVP-independent production of AQP-2 in decompensated liver cirrhosis[30]. The regulation of AQP-2 may be modified by a number of hormones and bioactive substances, such as angiotensin Ⅱ, aldosterone, dopamine, atrial natriuretic peptide, PGE_2 and adrenergic hormones[30]. The lack of responsiveness of the collecting ducts to changes in the plasma AVP could explain why a number of patients respond poorly to vaptans[30].

TREATMENT BY VASOPRESSIN RECEPTOR ANTAGONIST

Given the central role of AVP in limiting renal water excretion, AVP receptor antagonists represent a physiologic and rational method to increase renal water excretion[43]. Several vasopressin receptor antagonists have been evaluated in treating hyponatremia in patients with cirrhosis/end-stage liver disease[53]. These include the intravenous dual V1A/V2 -receptor antagonist conivaptan, and the oral V2 -receptor antagonists lixivaptan, RWJ-351647, satavaptan, and tolvaptan[53]. Main characteristics of randomized double-blind placebo-controlled trials on vaptans for patients with liver cirrhosis are listed in Table 1.

Lixivaptan

Guyader *et al*[54] first reported the randomized double-blind, placebo-controlled trial on the effect of V2-receptor antagonist lixivaptan (VPA-985; ascending single doses of 25, 50, 100, 200, and 300 mg) in patients with liver cirrhosis and ascites and found a dose-related increase in daily urine output and a dose-related decrease in urine osmolality together with increases in serum osmolality and Na concentration for 24 h[54]. Gerbes *et al*[55] studied the effects of lixivaptan (100 or 200 mg/d) or placebo in cirrhotics with ascites and hyponatremia (serum Na 115-132 mmol/L) in a double-blind controlled trial. The authors found that normalization of serum Na concentration was achieved in 27% and 50% of patients in the lixivaptan 100 mg/d and 200 mg/d groups, respectively, but in none of the patients in the placebo group. Although the effect of lixivaptan on ascites was not described in the report, a significant decrease of body weight was recorded in subjects receiving lixivaptan 200 mg/d[55]. Wong *et al*[56] investigated the add-on effects of lixivaptan (doses of 25, 125, and 250 mg twice daily) or placebo to diuretics (mostly furosemide and spironolactone) on cirrhotics with ascites and hyponatremia (< 130 mmol/L) and found that lixivaptan produced a significant overall aquaretic response with significant dose related increases in free water clearance and serum Na.

Lixivaptan was also shown to be effective and safe for long-term management of hyponatremia after outpatient initiation[2,57]. Normalization of serum Na levels was maintained during the 24-wk study period. It was titrated safely in the outpatient setting without over-rapid serum Na level correction[2,57].

RWJ-351647

Thuluvath *et al*[58] evaluated the effect of another selective V2-receptor antagonist RWJ-351647 in cirrhotics with ascites, who failed to lose ⩾ 2 kg weight in the week by the diuretic regimen of furosemide (40 mg/d) and spironolactone (100 mg/d), as a randomized double-blind, placebo-controlled trial. They found that this vaptan is an effective aquaretic causing dose-dependent increases in urine output and free water clearance, when co-administered with conventional diuretics[58]. There was no influence on the pharmacokinetics of concomitant furosemide and spironolactone in their study[58].

Satavaptan

Gines *et al*[59,60] investigated the effects of satavaptan on ascites management and serum Na in decompensated cirrhotic patients with and without hyponatremia. They first compared the effect of three fixed doses of satavaptan (5, 12.5 or 25 mg once daily) versus placebo for 14 d in 110 cirrhotic patients with ascites and hyponatremia (serum Na ⩽ 130 mmol/L), who continuously received spironolactone at 100 mg/d[59]. The authors reported that satavaptan was effective for control of ascites (indicated by a reduction in body weight and abdominal girth), which was associated with improvements in serum Na and concluded that this vaptan improves the control of ascites and hyponatremia in patients with cirrhosis under diuretic treatment[59]. They next evaluated the effect of satavaptan on ascites in 148 cirrhotics without hyponatremia as the similar double-blind randomized-controlled trial[60]. Duration of treatment was also 14 d but all patients received spironolactone 100 mg/d plus furosemide 20-25 mg/d. As a result, the administration of satavaptan was associated with reduction of ascites in cirrhotics without hyponatraemia[60].

On the other side, Wong *et al*[61] evaluated the long-term effect of satavaptan on the recurrence of ascites and survival. They first performed a double-blind placebo-controlled trial investigating the effects of the addition of satavaptan (5, 12.5 or 25 mg) or placebo to 100 mg spironolactone for 12 wk on ascites recurrence after a large volume paracentesis in 151 patients with liver cirrhosis irrespective of the presence of hyponatraemia[61]. Although the frequency of pa-

Table 1 Main characteristics of randomized double-blind, placebo-controlled trials on vaptans for patients with liver cirrhosis

Ref.	V2RA	Dose	Additional diuretics	Max treatment duration	No of patients vaptan(control)	Efficacy outcome	Main results	
Guyader *et al*[54]	Lixivaptan	25, 50, 100, 200, 300 mg/d	None (withheld for 48 h)	24 h	22 (5)	Daily urine output, urine osmolality, serum osmolality, serum Na	A significant dose-related increase in daily urine output and a dose-related decrease in urine osmolality together with significant increases in serum osmolality, Na, and vasopressin levels	
Gerbes *et al*[55]	Lixivaptan	100, 200 mg/d	Yes? (no detailed information available)	7 d	40 (20)	Serum sodium concentration, urine osmolality, body weight	Normalization of serum Na 27% (100 mg/d group) 50% (200 mg/d group); a significant reduction in urine osmolality and body weight	Fluid intake was restricted to 1000 mL/d
Wong *et al*[56]	Lixivaptan	50, 250, 500 mg/d	Yes	8 d	25 (8)	Free water clearance, serum sodium	Significant dose related increases in free water clearance and serum Na without changes in orthostatic blood pressure and serum creatinine levels	Fluid intake was restricted to 1500 mL/d
Schrier *et al*[63]	Tolvaptan	15, 30, 60 mg/d	Yes	30 d	63 (57) Salt 1 and Salt 2 study including other causes of hyponatremia	Serum Na	Effective in increasing serum Na concentrations at day 4 and day 30	
Thuluvath *et al*[58]	RWJ-351647	1, 2, 5 mg (single oral doses)	Spironolactone 100 mg/d + furosemide 40 mg/d	24 h	18 (6)	Urine volume, free water excretion, urine osmolality	Increases in cumulative urine volume and free water excretion, and a decrease in urine osmolality were noted in a dose-dependent manner reaching the statistical significance at the 5-mg dose	No changes in either serum chemistry or plasma AVP and renin levels
Ginès *et al*[59]	Satavaptan	5, 12.5, 25 mg/d	Spironolactone 100 mg/d	14 d	82 (28)	Body weight, abdominal girth, serum Na	Reduction in body weight and abdominal girth with improvements in serum Na	
Ginès *et al*[60]	Satavaptan	5, 12.5, 25 mg/d	Spironolactone 100 mg/d + furosemide 20-25 mg/d	14 d	113 (35)	Change in body weight The percentage of patients with a weight loss > 2 kg	Significant reduction in body weight; percentage of patients with a weight loss > 2 kg was greater	
Wong *et al*[61]	Satavaptan	5, 12.5, 25 mg/d	Spironolactone 100 mg/d	12 wk	115 (36)	Prevention of recurrent ascites after LVP (1) median time to first paracentesis (2) frequency of paracenteses (3) mean increase in ascites	Significant decrease in the frequency of paracenteses; No significant difference of mean increase in ascites	
Cárdenas *et al*[64]	Tolvaptan	15, 30, 60 mg/d	Spironolactone < 200 mg/d + furosemide < 80 mg/d	30 d	63 (57)	Serum Na (average daily area under the curve for serum Na); mental component summary scores of the SF-12 health survey	Improvement in serum Na levels and patient-reported health status without severe adverse effects	Sub-analysis of the SALT-1 and SALT-2 trials
Wong *et al*[62]	Satavaptan	5, 10 mg/d	Study 1 (None); Study 2 (one or more diuretics); Study 3 (withheld during the first 12 wk)	52 wk	720 (478)	Prevention of recurrent ascites after LVP (1) cumulative number of LVPs during the first 12 wk (2) reccurrence of ascites, defined as LVP and/or weight increase of > 4 kg (3) cumulative increase in ascites estimated	Not more effective than placebo in the control of ascites in any of the populations studied as estimated by the primary efficacy endpoints; slight advantages noted in delaying ascites formation and improvement of serum Na concentration in patients with hyponatremia	(Study 3) a higher rate of all-cause mortality, mostly associated with complications of cirrhosis in combination with diuretics
Okita *et al*[67]	Tolvaptan	7.5, 15 or 30 mg/d	Furosemide ⩾ 40 mg/d + spironolactone ⩾ 25 mg/d or furosemide ⩾ 20 mg/d + spironolactone ⩾ 50 mg/d	7 d	77 (27)	Body weight, abdominal circumference	7.5-30 mg/d reduced body weight and abdominal circumference 7.5 mg/d showed the maximum changes together with preferable tolerability	

Sakaida *et al*[68]	Tolvaptan	7.5 mg/d	furosemide ⩾ 40 mg/d + spironolactone ⩾ 25 mg/d or furosemide ⩾ 20 mg/d + spironolactone ⩾ 50 mg/d	7 d	84 (80)	Change in body weight from baseline; changes in abdominal circumference and ascites volume: improvement rates of lower limb edema and ascites-related clinical symptoms	change in body weight; -0.44 kg in the placebo group *vs* -1.95 kg in the tolvaptan group; higher improvement rates of limb edema and ascites-related clinical symptoms	Improve hyponatremia and derease body weight regardless of serum albumin level

All major studies are summarized in this table. In general, short-term effects of vaptans on hyponatremia and ascites are evident. Long-term effects are still controversial.

racentesis was decreased significantly in all satavaptan groups versus placebo, no significant difference of mean increase in ascites was found among the groups[61]. They further evaluated the efficacy and safety of satavaptan in three different populations of patients with cirrhosis and ascites by large-scale randomized, placebo-controlled trials[62]. They included 1200 patients in three studies comparing satavaptan with placebo in uncomplicated ascites (study 1) and difficult-to-treat ascites, with and without concomitant diuretic treatment (studies 2 and 3). They reported that satavaptan was not more effective than placebo in the control of ascites in any of the populations studied as estimated by the primary efficacy endpoints: worsening of ascites (study 1) and the cumulative number of large-volume paracentesis during 12 wk (studies 2 and 3)[62]. However, as for secondary efficacy endpoints, slight advantages of satavaptan over placebo were noted in delaying ascites formation and improving serum Na concentration in patients with hyponatremia[61]. They finally concluded that satavaptan, alone or in combination with diuretics, is not clinically beneficial in the long-term management of ascites in cirrhosis[62]. Moreover, when satavaptan was administered in combination with diuretics to prevent ascites recurrence after large-volume paracentesis (study 3), a higher rate of all-cause mortality, mostly associated with known complications of cirrhosis, was recorded during the 52 wk of follow-up[62]. These limited efficacy and safety concerns resulted in withdrawal of the drug by the pharmaceutical company[33,53].

Tolvaptan

Shrier *et al*[63] reported the effect of another V2-receptor antagonist tolvaptan for 448 patients with hyponatremia of variable etiology as two double-blind placebo-controlled studies (Study of Ascending Levels of Tolvaptan in Hyponatremia1 and 2 [SALT-1 and SALT-2]). The subjects included 138 patients with congestive heart failure, 190 patients with SIADH and 120 patients with liver cirrhosis[63]. The authors did not evaluate the results separately based on etiology, but simply showed that 15 mg of oral tolvaptan (increased to 30 or 60 mg if needed) was effective in increasing serum Na concentrations in these patients with euvolemic or hypervolemic hyponatremia[63]. Cárdenas *et al*[64] then performed sub-analysis of these SALT-1 and SALT-2 trials evaluating the efficacy and safety of tolvaptan in patients with cirrhosis and hyponatremia and found improvement in serum Na levels and patient-reported health status without severe adverse effects[64]. Additionally, hyponatremia recurred in tolvaptan-treated patients after discontinuation of tolvaptan[64].

The SALTWATER trial, an open-label extension of SALT 1 and SALT 2, enrolled 111 individuals including 20 cirrhotics to evaluate whether tolvaptan remained safe and effective during long-term use[2,65]. Initial dose was 15 mg, which was increased to attain a normal serum Na level[2,65]. At 50 wk, the serum Na concentration normalized in approximately 60% of patients[41,65]. During the more than 4-year study period, only one required withdrawal from the study. As in the short-term trials, there were no reported adverse effects concerning for osmotic demyelination[2,65].

In a Japanese pilot study[66], tolvaptan was administrated at titrated doses of 15, 30, and 60 mg once daily for 3 d at each dose to 18 cirrhotic patients with persistent ascites and/or lower limb edema despite receiving oral furosemide at 40 mg/d or higher. In this study, tolvaptan was proved to decrease body weight and abdominal circumference dose-dependently and to improve ascites and edema beginning from 15 mg[66]. Japanese study group then designed a 7-d multicenter double-blind trial of tolvaptan to determine the optimal dose for hepatic edema[67]. One hundred four participants were stratified randomly to four groups receiving tolvaptan at 7.5, 15 or 30 mg/d, or placebo as an add-on to conventional diuretics for 7 d. The subjects selected were poor responders to the standard daily dose of concomitant diuretics: (1) a loop diuretic at a daily dose equivalent to furosemide 40 mg/d or higher and spironolactone at 25 mg/d or higher; or (2) a loop diuretic at a daily dose equivalent to furosemide 20 mg/d or higher and spironolactone at 50 mg/d or higher[67]. Although tolvaptan at 7.5-30 mg/d reduced body weight and abdominal circumference compared with placebo, dose of 7.5 mg/d showed the maximum changes together with preferable tolerability[67]. From these results, 7.5 mg/

d was considered the optimal dose in patients with liver cirrhosis and ascites, who showed inadequate response to conventional diuretics in Japan[67]. This conclusion was verified by another multicenter double-blind placebo-controlled trial by Sakaida *et al*[68], by which tolvaptan is proved to improve hyponatremia and to exert its effect on body weight and initial urine volume of the patients regardless of serum albumin level[68,69]. They also doubled the duration of tolvaptan treatment and showed that it's effect on body weight persisted for 14 d[70]. In order to avoid adverse effects of tolvaptan in cirrhotics, Sakaida *et al*[71] further tried to decrease the dosage of tolvaptan and compared 3.75 mg/d and 7.5 mg/d schedules by a double-blind, parallel-group study. Although tolvaptan resulted in dose-dependent decreases in body weight and ascites volume and increases in urine output, 3.75 mg/d tolvaptan exerted significant effects as well[71].

Conivaptan

In patients with Child-Pugh class A-C cirrhosis, conivaptan is administered *via* an intravenous loading dose of 10 mg followed by continuous infusion of 10 mg over 24 h for 2 to 4 d with titration up to 20 mg over 24 h if serum Na is not rising at the desired rate[53].

A retrospective review of 24 cirrhotic patients with hyponatremia showed that it raised serum Na in patients with and without diuretics[53,72]. Because conivaptan is available only as a parenteral formulation, chronic use in cirrhotic patients with hyponatremia may be limited. Theoretically, it should be cautious to use conivaptan in patients with cirrhosis because it blocks both V2 and V1 A receptors[53,73]. There is a possibility that V1A inhibition may result in splanchnic vasodilation, which leads to further reduction in blood pressure and increased risk of variceal bleeding[33]. Dilatation of the splanchnic bed and interference with platelet aggregation could exacerbate complications of variceal bleeding[41].

AREAS OF UNCERTAINTY

It is not determined if V2-receptor antagonists are helpful for asymptomatic hyponatremic patients[43]. It is also undetermined if early use of vaptans prevent diuretic-induced hyponatremia in cirrhotics. Although it remains unclear whether correction of the hyponatremia per se will improve patient outcomes, V2-receptor antagonist offer an opportunity to test this uncertainty in patients with euvolemic and hypervolemic hyponatremia[43].

So far no study has revealed that the effects of vaptans are related to plasma AVP levels in patients with liver cirrhosis. As plasma AVP levels are considered to be normal in cirrhotics without hyponatremia, the reported effects of tolvaptan in these patients may be related to hypersensitivity of V2-receptor to AVP as noted in congestive heart failure[74]. In fact, the effect of spironolactone for cirrhotics with normal plasma aldosterone levels have been explained by the renal tubular hypersensitivity to aldosterone[75].

Proper indication of vaptans

Hospitalized patients with mild to moderate symptoms of hyponatremia can be considered ideal candidates for the use of vaptans. Although restriction of fluid intake has been recommended in patients with severe hyponatremia, it has very limited efficacy for cirrhotic patients[1]. While it remains speculative as to whether correction of the hyponatremia per se will improve patient outcomes, the vaptans offer an opportunity to test this uncertainty in patients with euvolemic and hypervolemic hyponatremia[43]. One remaining question is if they are helpful in hyponatremic patients who are asymptomatic or mildly symptomatic[43]. Hyponatraemia with serum Na $\leqslant$ 130 mEq/L is one of several predictive factors for the development of overt hepatic encephalopathy[44,76]. Investigators have hypothesized that low-grade cerebral edema associated with hyponatremia may predispose cirrhotics to encephalopathy[53,77]. Treating hyponatremia is, therefore, considered to be a prudent step to reduce the frequency and severity of encephalopathy in cirrhosis[41]. Hyponatraemia in cirrhosis is associated with impaired cognition and poor health-related quality of life (HRQOL), which were recently proved to be improved by hyponatraemia correction by short-term tolvaptan therapy[78].

Dahl *et al*[79] made meta-analyses to evaluate the effects of vaptans (tolvaptan, satavaptan and lixivaptan) on patients with cirrhosis and hyponatraemia or ascites. As a result, they did not find clear differences between the vaptan groups and the control groups regarding mortality, variceal bleeding, hepatic encephalopathy, spontaneous bacterial peritonitis, hepatorenal syndrome, or renal failure, although vaptans increased serum Na levels and led to reductions in weight and the time to the first paracentesis. They admitted that vaptans have a small beneficial effect on hyponatremia and ascites but concluded that these data do not support the routine use of vaptans in cirrhosis[79]. Further studies about using different end-points such as hospitalizations for hyponatremia, need for more or less diuretics to control the ascites, and need for paracentesis, would better define how to use this new class of drugs in the patient with cirrhosis and ascites[80].

A recent FDA drug safety communication recommended that tolvaptan should not be used for longer than 30 d and should not be used in patients with underlying liver disease due to a risk of liver injury leading to liver transplant or death[81]. This arose from surveillance in a 3-year placebo-controlled study of 1400 patients with autosomal dominant polycystic kidney disease. Among them 3 patients treated with tolvaptan at a dose of

120 mg/d developed significant increases in serum ALT and total serum bilirubin levels[81]. No vasopressin receptor antagonist is currently approved by the FDA for treatment of hyponatremia in patients with liver disease or cirrhosis[33].

Proper doses of vaptans

The effect of vaptans may be less robust in hyponatremia of cirrhosis compared with other causes[56,63,65]. A subgroup analysis of cirrhotic patients in the SALT trials also supported this observation[2]. Reasons for limited response to vaptans in some hyponatremic patients with advanced cirrhosis may be that avid proximal reabsorption of solute leads to decreased distal delivery of the glomerular filtrate, although this possibility has not been studied. Another possibility is that V2 receptor-independent pathways play a role in APQ-2 regulation in cirrhosis[2,30]. The difference in the doses of tolvaptan between international trials and Japanese trials are noticeable. Of course, it should be kept in mind that the primary efficacy outcomes in the Japanese studies were improvement of ascites and edema of the extremities and not that of severe hyponatremia, hence refractory ascites in end-stage cirrhotics was excluded from these studies. International tolvaptan trials used 15 to 60 mg/d in cirrhotics for decompensated cirrhotics with hyponatremia, whereas the doses of Japanese trials first settled between 7.5 to 30 mg/d[66] finally decreased to 3.75 and 7.5 mg for decompensated cirrhotics without hyponatremia[71]. This study, however, showed that tolvaptan induced significant increases in urine volume and significant decreases in body weight from basal levels for 4 d even in the 3.75 mg/d group[71]. Significant increase in serum Na concentrations within normal limits were noted in the 7.5 mg/d group[71]. Although the approved dose of tolvaptan for cirrhotics is 7.5 mg/d, intial dose of 3.75 mg/d is recommended for safety in advanced cirrhotics in Japan. Although the reason why some Japanese cirrhotic patients responded to such a low dose of tolvaptan as 3.75 to 7.5 mg/d is not clear, it is now advisable to use low-dose tolvaptan for cirrhotic ascites to prevent the risky liver injury warned by FDA[33].

Effect for refractory ascites

Zhang *et al*[82] recently reported that the combination of 15 mg/d tolvaptan with diuretics effectively increased the urine output in 89.7% of patients with refractory ascites. They selected the patients who were not satisfactorily controlled after either 1 wk of Na intake restrictions, albumin infusion and high doses of diuretics (more than 160 mg/d of furosemide and 200 mg/d of spironolactone) or 2 wk of large volume therapeutic paracentesis.

Patients with Child-Pugh scores of greater than 10 have been excluded in most tolvaptan trials except for this study[82]. The more severe forms of hyponatremia (Na < 125 mmol/L) are seen in patients with more advanced liver disease, and hence the safety and efficacy of vaptans in this group of patients with cirrhosis should be carefully examined[80]. It is conceivable that combining vaptans with diuretics could be beneficial in patients with refractory ascites reducing the frequency of large-volume paracentesis[28]. This hypothesis, however, was not validated with large-scale randomized controlled studies. We cannot generally recommend vaptans for all refractory ascites, until they are proved to be effective in the same kind of study that include only patients with refractory ascites.

A low serum Na level is a strong predictor of pre-transplant mortality, independent of the Model for End-stage Liver Disease score (MELD)[83]. Vaptans may have some merit in the management of patients before liver transplantation. This should be also evaluated in an adequate control study.

FUTURE POSSIBILITY OF ASCITES TREATMENT BY DIURETICS AND VAPTANS

The doses of diuretics in combination with vaptans have been reported to be variable; *i.e.,* furosemide 20-80 mg/d and spironolactone 25-200 mg/d.

Large-doses of diuretics frequently lead difficult-to-treat hyponatremia, which disturbs further use of diuretics. Although the level of hyponatremia at which diuretics should be abandoned is contentious, most experts agree that they should temporarily stop diuretics in patients whose serum Na decreases to less than 120-125 mmol/L[25]. By an addition of vaptans we may continue diuretics to these severe hyponatremic patients with ascites, or we may prevent the development of severe hyponatremia itself in the diuretic treatment. It is advisable to evaluate in a prospective controlled study if we can decrease the incidence of diuretic-intractable ascites by an early combination of diuretics and vaptans. The fact that disturbed water excretion already exists in the early cirrhosis without hyponatremia may become the theoretical basis for this strategy.

Considering the mechanism of water excretion, markedly decreased GFR and distal delivery of fluid may cancel the effect of vaptans. Although we have no definite data yet, the effect of tolvaptan seems weak in patients with renal dysfunction related to the underfilling state. Except for Zhang *et al*[82]'s report, we have no evidence to say that vaptans benefit these advanced cases. More clinical data are needed to know if tolvaptan is really effective for difficult-to-treat ascites in patients with marked underfilling state. Although vaptans show much promise, further study is needed to clarify whether we can establish this

drug in the outpatient setting as a combination with diuretics[80] and whether we can improve the morbidity and cost burden[2].

CONCLUSION

Although there is no evidence that correcting the serum Na influences the patient's prognosis, it is clear that severe hyponatremia leads to hospitalization, discontinuation of diuretics and fluid restriction, all of which are undesirable outcomes[80]. Even if hyponatremia is not the direct cause of symptoms, it may lower the threshold for changes in mental status resulting from poor cerebral perfusion[43]. Therefore, meticulous use of vaptans may become a choice in the management of ascites and hyponatremia before considering large-volume paracentesis or TIPS. Although tolvaptan has a possibility to make a breakthrough in the treatment of difficult-to-treat ascites, its high price is a major barrier to go beyond for the future[2].

Considering that the use of vaptans is only a symptomatic therapy, we should make every effort to improve the backgrounds of water retention. There is now considerable evidence to suggest that long-term anti-HBV treatment can improve liver fibrosis in patients with advanced hepatitis B virus-related cirrhosis[84]. As endotoxemia and resultant increase of nitric oxide are important precipitating factors for the underfilling state of cirrhotics, adequate management of the gut-liver axis may merit the patients[85].

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Gut microbiota and host metabolism in liver cirrhosis

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Abstract

The gut microbiota has the capacity to produce a diverse range of compounds that play a major role in regulating the activity of distal organs and the liver is strategically positioned downstream of the gut. Gut microbiota linked compounds such as short chain fatty acids, bile acids, choline metabolites, indole derivatives, vitamins, polyamines, lipids, neurotransmitters and neuroactive compounds, and hypothalamic-pituitary-adrenal axis hormones have many biological functions. This review focuses on the gut microbiota and host metabolism in liver cirrhosis. Dysbiosis in liver cirrhosis causes serious complications, such as bacteremia and hepatic encephalopathy, accompanied by small intestinal bacterial overgrowth and increased intestinal permeability. Gut dysbiosis in cirrhosis and intervention with probiotics and synbiotics in a clinical setting is reviewed and evaluated. Recent studies have revealed the relationship between gut microbiota and host metabolism in chronic metabolic liver disease, especially, non-alcoholic fatty liver disease, alcoholic liver disease, and with the gut microbiota metabolic interactions in dysbiosis related metabolic diseases such as diabetes and obesity. Recently, our understanding of the relationship between the gut and liver and how this regulates systemic metabolic changes in liver cirrhosis has increased. The serum lipid levels of phospholipids, free fatty acids, polyunsaturated fatty acids, especially, eicosapentaenoic acid, arachidonic acid, and docosahexaenoic acid have significant correlations with specific fecal flora in liver cirrhosis. Many clinical and experimental reports support the relationship between fatty acid metabolism and gut-microbiota. Various blood metabolome such as cytokines, amino acids, and vitamins are correlated with gut microbiota in probiotics-treated liver cirrhosis patients. The future evaluation of the gut-microbiota-liver metabolic network and the intervention of these relationships using probiotics, synbiotics, and prebiotics, with sufficient nutrition could aid the development of treatments and prevention for liver cirrhosis patients.

Key words: Liver cirrhosis; Microbiota; Metabolism; Fatty acids; Probiotics

Core tip: The gut microbiota has the capacity to produce a diverse range of compounds that have a major role in regulating the activity of distal organs and the liver is strategically positioned downstream of the gut indicating the importance of the gut-liver axis. This review focuses on gut microbiota and host metabolism in liver cirrhosis. The serum lipid levels of phospholipids, free fatty acids, eicosapentaenoic acid, arachidonic acid, and docosahexaenoic acid have significant correlations with specific fecal flora in liver cirrhosis. Various blood metabolome such as cytokines, amino acids, and vitamins are correlated with gut microbiota in probiotics-treated liver cirrhosis patients.

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INTRODUCTION

An increasing amount of recent evidence has demonstrated that several diseases, such as irritable bowel syndrome, inflammatory bowel disease, diabetes, allergy, cancer, obesity, autism and liver disease, are related to alterations in intestinal microbiota (known as dysbiosis)[1-3]. Gut-derived complications in liver cirrhosis such as small intestinal bacterial overgrowth and increased intestinal permeability (leaky gut), resulting in bacterial or endotoxin translocation-related systemic disorders such as spontaneous bacterial peritonitis, hyperdynamic state, portal hypertension, hepatorenal syndrome, hepatic encephalopathy, and multiple organ failure, have been reported in clinical settings[2,4-8]. Different etiologies of liver cirrhosis, including viral hepatitis, alcoholic liver disease (ALD), and non-alcoholic fatty liver disease (NAFLD), have different gut microbiota and mechanisms of developing liver fibrosis. Furthermore, each disease has a different hepatic metabolism, suggesting further development in this research area[2,7-9]. However, there have been few reports of correlations between gut microbiota and host metabolism in cirrhotic patients. This review will discuss (1) the relationship between gut microbiota and host metabolism in general; (2) the results of intervention for liver cirrhosis by probiotics; and (3) gut-microbiota and host metabolism in cirrhosis and the use of systems biology as a tool for analysis.

GUT MICROBIOTA AND HOST METABOLISM

The anatomy of the liver provides its close interaction with the gut[9,10]. Gut-derived bacteria and their components and metabolites, as well as nutrients and other signals are delivered to the liver *via* the portal circulation. Then, the liver plays a crucial role in defense against gut-derived materials, which is defined as the gut-liver axis[9,10]. Gut microbiota function as a bioreactor for autonomous metabolic and immunological functions that can mediate responses within the host environment in response to external stimuli[11]. The complexity of the gut microbiota suggests that it behaves as an organ. Therefore, the concept of the gut-liver axis must be complemented with the gut-microbiota-liver network because of the high intricacy of the microbiota components and metabolic activities[11].

The host and its gut microbiota coproduce a large array of small molecules during the metabolism of food and xenobiotics (compounds of non-host origin that enter the gut with the diet or are produced by microbiota), many of which play critical roles in communication between host organs and the host's microbial symbionts. The metabolite, gut microbiota, and potential biologic functions are shown in Table 1[12-40].

Short-chain fatty acids (SCFAs), predominantly butyrate, acetate and propionate, are anaerobically produced by gut microbiota in the intestine. SCFAs, particularly butyrate, are a significant source of energy for gut enterocytes, and influence the gastrointestinal barrier function through the stimulation of tight junction and mucous production[41-43]. The authors showed that tight junction permeability was decreased by SCFAs in a Caco-2 intestinal monolayer and human umbilical vein endothelial cell monolayer, *via* lipoxygenase activation in *in vitro* studies[44,45]. This suggests that SCFAs may have biological effects in other organs as well as the gastrointestinal tract. Furthermore, there is growing evidence to suggest a role for SCFAs in reducing inflammation[41]. Our previous reports showed that increased pro-inflammatory cytokine production and nuclear factor kappa B activity induced by lipopolysaccharide (LPS) were downregulated by SCFAs using human peripheral blood mononuclear cells and co-culture of macrophages and adipocytes[46-48]. LPS-induced acute liver injury was attenuated by orally administered tributyrin, a prodrug of butyrate and a dairy food component, *via* increased portal vein concentration up to one to two orders of magnitude in rats[49]. In humans, two reports by Bloemen *et al*[50,51] measuring portal and hepatic venous SCFA concentrations indicated a porto-systemic shunting effect in liver cirrhosis patients.

SCFAs are also proposed to increase satiety following the consumption of a diet rich in fiber as they act as agonists for free fatty acid receptors 2 and 3 (FFAR2/3 known as G-protein coupled receptor; GPR43/41). Both of these GPRs trigger the production and release of glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and other gut hormones that stimulate satiety in the host[52]. Gut intestinal (GI) hormones such as ghrelin and leptin secretion are mediated on enteroendocrine cells by the action of SCFAs[18,53].

Table 1 Metabolite, gut microbiota, and potential biologic functions

Metabolites	Related bacteria	Potential biological functions	Ref.
Short-chain fatty acids	Clostridial clusters IV and XIVa of *Firmicutes*, including species of *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus*	Decreased colonic pH, inhibit the growth of pathogens; stimulate water and sodium absorption; participate in cholesterol synthesis; provide energy to the colonic epithelial cells; GI hormones secretion *via* enteroendocrine cells, implicated in human obesity, insulin resistance and type 2 diabetes, colorectal cancer. Immunological homeostasis in the gut	[14-18]
Bile acids	*Lactobacillus, Bifidobacteria, Enterobacter, Bacteroides, Clostridium*	Absorb dietary fats and lipid-soluble vitamins, facilitate lipid absorption, maintain intestinal barrier function, signal systemic endocrine functions to regulate triglycerides, cholesterol, glucose and energy homeostasis	[19-21]
Choline metabolites	*Faecalibacterium prausnitzii, Bifidobacterium*	Modulate lipid metabolism and glucose homeostasis. Involved in nonalcoholic fatty liver disease, dietary induced obesity, diabetes, and cardiovascular disease	[22,23]
Phenolic, benzoyl, and phenyl derivatives	*Clostridium difficile, F. prausnitzii, Bifidobacterium, Subdoligranulum, Lactobacillus*	Detoxification of xenobiotics; indicate gut microbial composition and activity; utilize polyphenols. Urinary hippuric acid may be a biomarker of hypertension and obesity in humans. Urinary 4-hydroxyphenylacetate, 4-cresol, and phenylacetate are elevated in colorectal cancer. Urinary 4-cresyl sulfate is elevated in children with severe autism	[24,25]
Indole derivatives	*Clostridium sporogenes, E. coli*	Protect against stress-induced lesions in the GI tract; modulate expression of proinflammatory genes, increase expression of anti-inflammatory genes, strengthen epithelial cell barrier properties. Implicated in GI pathologies, brain-gut axis, and a few neurological conditions	[26-28]
Vitamins	*Bifidobacterium*	Provide complementary endogenous sources of vitamins, strengthen immune function, exert epigenetic effects to regulate cell proliferation	[29,30]
Polyamines	*Campylobacter jejuni, Clostridium saccharolyticum*	Exert genotoxic effects on the host, anti-inflammatory and antitumoral effects. Potential tumor markers	[31,32]
Lipids	*Bifidobacterium, Roseburia, Lactobacillus, Klebsiella, Enterobacter, Citrobacter, Clostridium*	Impact intestinal permeability, activate intestine-brain-liver neural axis to regulate glucose homeostasis; LPS induces chronic systemic inflammation; conjugated fatty acids improve hyperinsulinemia, enhance the immune system and alter lipoprotein profiles. Cholesterol is the basis for sterol and bile acid production	[33,34]
Neurotransmitters and neuroactive compounds: serotonin, tryptophan, kynurenine. dopamine, noradrenaline, GABA	*Lactobacillus ,Bifidobacterium, Escherichia, Bacillus, Saccharomyces, Candida, Streptococcus, Enterococcus*	Neurofunction related as mood, emotion, cognition, reward (CNS), motility/ secretion and behavior	[35-39]
HPA hormones: cortisol	*Lactobacillus, Bifidobacterium*	Indirect regulation of HPA. Regulation of stress response, host metabolism, anti-inflammation, wound healing, endocrine abnormalities prominent in stress related psychiatric disorders	[40]

GI: Gastrointestinal; LPS: Lipopolysaccharide; GABA: γ-aminobutyric acid; CNS: Central nervous system; HPA: Hypothalamic-pituitary-adrenal. Adapted from Ref. [12, 13] and revised by the authors.

SCFAs that traffic to distal sites and can be carried by monocarboxylate transporters, which are abundantly expressed at the blood-brain barrier then enter the central nervous system[54-57]. However, it remains to be definitively established whether alterations in intestinal microbiota-derived SCFAs are actually reflected at physiologically relevant concentrations in the central nervous system[13].

Bile is composed of individual bile acid moieties, mucous, phospholipids and biliverdin, and their main physiological roles in the small intestine are the emulsification of fats, the release of fat-soluble vitamins and regulation of cholesterol metabolism[58]. Specific bile acids differentially act as ligands to activate or repress host receptors, including farnesid X receptor, pregnane X receptor, vitamin D receptor and the GPR, TGR5. These receptors are expressed locally on various intestinal epithelial cells and systematically, within a diverse range of organs including both the liver and adipose tissue[21]. Therefore, bile acids function as systemic signaling molecules and significantly alter host gene-expression profiles[21,59].

Choline synthesized by intestinal biota is important for lipid metabolism and is metabolized to trimethylamine, then further metabolized in the liver to trimethylamine-N-oxide that contributes to the development of cardiovascular disease[22]. Reducing the bioavailability of choline can contribute to NAFLD and altered glucose metabolism[60]. Phenolic, benzoyl, and phenyl derivatives produced by the detoxification of xenobiotics have various bioactivities, are indicators of microbial composition and activity, and are useful biomarkers for several diseases including liver disease[25].

A significant amount of the neurotransmitter dopamine is produced in the human gut[61]. Norepinephrine and dopamine production in the gut is mediated by the expression of β-glucuronidases from commensal gut bacteria through the cleavage of their inactive conjugated forms[62]. Nitric oxide produced by gut microbes plays a pivotal role in gastric emptying[63]. The

inhibitory transmitter γ-aminobutyric acid is generated by *Lactobacillus brevis* and *Bifidobacterium dentium*, both of which have been isolated from humans[64,65]. The precursors to neuroactive compounds, such as tryptophan for serotonin function and the kynurenine pathway, are controlled by gut-microbiota as a bidirectional communication component of the brain-gut axis[39].

The role of gut microbiota in the development of the hypothalamic-pituitary-adrenal (HPA) axis has been extensively analyzed using germ-free mice[40]. The concept of Microbial Endocrinology was reported and the importance of controlling gut microbiota in relation with various host functions was discussed by Lyte and colleagues[13,66].

GUT MICROBIOTA AND ITS MANIPULATION IN LIVER CIRRHOSIS

The gut microbiota plays an important role in the pathophysiology of cirrhosis. Changes in gastrointestinal functions, including malabsorption and small intestinal bacterial overgrowth, is common with concomitant portal hypertension in cirrhosis patients[67]. Recent reviews reported the pathophysiologic changes of gut microbiota in cirrhotic patients, gut-bacterial interactions, "leaky gut", translocation of bacteria and gut-derived LPS in infectious complications, spontaneous bacterial peritonitis, and hepatic encephalopathy[2,5,68,69]. Hyperdynamic circulation, portal hypertension, hepatic encephalopathy, renal disturbance including hepatorenal syndrome, and cirrhotic cardiomyopathy in cirrhosis are correlated with endotoxemia[6].

ALD is a spectrum of alcoholic diseases including steatosis, steatohepatitis, acute alcoholic steatohepatitis, alcoholic fibrosis, and cirrhosis (Laennec's Cirrhosis) caused by excessive alcohol use over a prolonged period of time[9]. Multiple pathogenic factors are involved in the development of ALD. Alcohol and its metabolites induce reactive oxygen species and hepatocyte injury through mitochondrial damage and endoplasmic reticulum stress[70,71]. The activation of Kupffer cells has been identified as a central element in the pathogenesis of ALD. Kupffer cells and recruited macrophages in the liver are activated by LPS through Toll-like receptor (TLR) 4, and LPS levels increase in the portal and systemic circulation after excessive alcohol intake[72]. Fibrosis is a dynamic and progressive process governed by stellate cell activation by LPS, TLR4 and inflammatory cytokines such as transforming growth factor-β signaling[73].

NAFLD is the most common cause of chronic liver disease worldwide as a result of the increasing prevalence of obesity, characterized by a spectrum of liver diseases ranging from simple fatty liver (NAFL) to steatohepatitis (NASH) with a possible progression to fibrosis[11]. The concept of the gut-liver axis may be complemented with the gut-microbiota-liver network because of the high intricacy of the microbiota components and metabolic activities; these activities form the active diet-driven power plant of the host[11,74]. However, there have been few descriptive studies on gut-microbiota composition under NASH and NAFLD conditions; therefore, the type and role of gut microbes in human liver damage are poorly understood[11]. The use of meta-omic platforms to assist the understanding of NAFLD gut-microbiota alteration as a tool and its application in patients has been proposed[11]. A detailed explanation of the meta-omic platform is described later.

As for the therapeutic approach to control dysbiosis, selective digestive decontamination (SDD), probiotics, prebiotics, and synbiotics have been performed. SDD is a method to treat bacterial translocation-related complications caused by poorly absorbed antibiotics such as quinolone. SDD was effective in some studies but a major concern of long-term antibiotic prophylaxis is the development of antibiotic-resistant bacteria and increased infections in chronic disease situations[75].

In this review, probiotics and synbiotics in liver cirrhosis are discussed. Probiotics were defined by the World Health Organization in 2001 as "live microorganisms, when administered in adequate amounts, confer a health benefit on the host". Prebiotics belong to a group of nondigestive food constituents that selectively alter the growth and/or activity of bacteria in the colon. The combined use of probiotics and prebiotics is called synbiotics. *Bifidobacteria* and *Lactobacilli*, the main species of probiotics, are considered as nonpathogenic to humans. The pathophysiologic basis for using probiotics in liver disease is as follows: (1) prevention of infection; (2) improvement of the hyperdynamic circulatory state of cirrhosis; (3) prevention of hepatic encephalopathy; (4) improvement of liver function; and (5) therapeutic potential of NAFLD[76].

Infection, such as spontaneous bacterial peritonitis and endotoxemia, can be induced in compensated and decompensated cirrhotic patients with or without surgery. Rayes *et al*[77,78] also reported the beneficial effects of probiotics against infectious complications in cirrhosis patients that underwent liver transplantation or liver resection. We reported that synbiotics (*Bifidobacterium*, *Lactobacillus*, and galacto-oligosaccharides) treatment attenuated the decrease in intestinal integrity as assessed by serum diamine-oxidase activity and reduced infectious complications after hepatic surgery[79]. Meta-analysis indicated an apparent reduction of infectious complications (odds ratio 0.24) in abdominal surgery[80]. However, a small size randomized controlled clinical trial showed that VSL#3® treatment to decompensated cirrhotic patients reduced plasma aldosterone, but did not reduce the incidence of spontaneous bacterial peritonitis[81]. As for hyperdynamic circulation, a prospective study reported that VSL#3® improved hemodynamic states, hepatic

Table 2 Effects of the probiotics intervention on gut microbiota composition and its clinical and/or biochemical consequences

Type of study	Category of patients/duration of treatment	Probiotics	Clinical outcome	Ref.
RCT	36 cirrhotics/6 mo	*Lactobacillus.acidophilus, Lactobacillus bulgaricus, Bifidobacterium lactis* and *S. thermophiles*	Blood ammonia levels	[88]
RCT	65 cirrhotics/6 mo	*Lactobacilli*	Incidence of HE, hospital admission, plasma-ammonia level, serum bilirubin level	[83]
R	50 cirrhotics/14 d	*Bifidobacterium, L. acidophilus* and *Enterococcus vs Bacillus subtilis* and *Enterococcus faecium*	*Bifidobacterium* count, fecal pH, fecal and blood ammonia in both groups, endotoxin level only with *B. subtilis* and *E. faecium*	[89]
RCT	17 cirrhotics with HVPG > 10 mmHg/2 mo	VSL # 3®	Plasma aldosterone	[81]
RCT	41 chronic liver disease/14 d	*Bifidobacterium bifidus, L. acidophilus, Lactobacillus bulgaricus*, and *S. thermophilus*	*E. coli* count, intestinal flora imbalance, improvement in debilitation, food intake, abdominal distension, and ascitic fluid	[90]
RCT	66 cirrhotics underwent liver transplantation/2 wk after the operation	*Pediacoccus pentosaceus, Leuconostoc mesenteroides, Lactobacillus paracasei* and *Lactobacillus plantarum*	Infectious complication	[78]
RCT	39 cirrhotics/42 d	*E. coli Nissle*	Endotoxemia, Child-Pugh score, Restoration of normal colonic colonization	[91]
RCT	63 cirrhotics patients with large oesophageal varices without history of variceal bleeding/2 mo	Propranolol plus VSL # 3®	HVPG, plasma TNF-α levels.	[92]
RCT	25 nonalcoholic minimal HE cirrhotics (defined by a standard psychometric battery)/60 d	Yogurt contained *L. bulgaricus* and *S. thermophilus*	Minimal HE	[93]
RCT	61 cirrhotics underwent hepatic surgery/2 wk before and after surgery	*Lactobacillus casei* strain Shirota, *Bifidobacterium breve* strain Yakult, and galactooligosaccharides	Intestinal integrity, infectious complication	[79]
RCT	63 cirrhotics underwent liver transplantation/12 d after the operation	*L. plantarum 299* and *oat fiber*	Infectious complication	[77]
RCT	50 cirrhotics underwent living donor liver transplantation/2 d and 2 wk before and after the operation, respectively	*L. casei* strain Shirota, *B. breve* strain Yakult, and galactooligosaccharides	Infectious complication	[94]
RCT	30 cirrhotics with minimal HE/4 wk	*Lactobacillus* GG	Endotoxemia, gut dysbiosis, gut microbiome-metabolome linkages	[69]
RCT	138 cirrhotics/3 mo	VSL # 3®	HE, small intestinal bacterial overgrowth	[84]

HE: Hepatic encephalopathy; HVPG: Hepatic venous pressure gradient; RCT: Randomized controlled trial; R: Randomized.

venous pressure gradients, cardiac index, heart rate, systemic vascular resistance, and mean arterial pressure, without any adverse reactions in cirrhosis patients with ascites[82]. Several reports showed that probiotics treatment to cirrhosis patients prevented hepatic encephalopathy with decreased blood ammonia or bilirubin levels[83,84]. The mechanism of hepatic encephalopathy prevention is the improvement of small intestinal bacterial overgrowth and gut dysbiosis[84]. VSL#3® treatment improved hepatic function, serum aspartate transaminase and alanine transaminase levels, both in NAFLD and cirrhosis patients with ALD and hepatitis C virus infection[85]. As for NAFLD, the immune-regulatory effects of probiotics may be beneficial in NAFLD treatment, and they should be considered a complementary therapeutic approach in NAFLD patients as indicated in a review by Ferolla *et al*[86]. The mechanisms of action are increased fatty acid formation, change in colonic pH, growth factor induction, change in intestinal flora, bacterial adherence inhibition by colonization resistance, immune modulation, increased phagocyte activity, increased IgA secretion and modulation of lymphocyte functions[87].

Table 2[69,77-79,81,83,85,88-94] shows the main randomized controlled studies in cirrhosis patients. These reports suggest that probiotics treatment improved gut dysbiosis and bacterial translocation, leading to the improvement of cirrhosis prognosis. Trials with probiotics in general have been limited by a lack of stability of the products as a drug and differences among bacterial species and subspecies[93,95]. Therefore, the results have been heterogeneous with regard to the duration, type of organism or combination of organisms and outcomes, and mixed results been achieved. The properties of different probiotic species vary and can be strain-specific. This is also complicated by a lack of uniformity in batch-to-batch formulations and studies not being performed under an investigational new drug regulatory procedure. The variety of available probiotics also makes the accumulation of evidence difficult. Furthermore, a risk of bacterial sepsis and fungal sepsis should be considered in infants and immune-deficient patients[96].

GUT MICROBIOTA AND HOST METABOLISM IN LIVER CIRRHOSIS

The effect of gut microbiota on host metabolism has been reported in the context of host-gut microbiota metabolic interactions in dysbiosis related metabolic diseases (diabetes, obesity and chronic liver disease) as various obesity-associated mechanisms including insulin resistance, fibrosis, and abnormalities in lipid metabolism[8,86,97]. However, few studies on the association between gut microbiota and metabolism in cirrhosis have been reported.

We previously reported the measurement of fecal microbiota, organic acids, and plasma lipids in hepatic cancer patients in three different groups characterized by histopathology as normal liver, chronic hepatitis/liver fibrosis, and liver cirrhosis[46]. These data were obtained by fecal culture without using probiotics and by comparison among different liver diseases. The serum lipid levels of phospholipids, free fatty acids, eicosapentaenoic acid (EPA), EPA/arachidonic acid (AA) ratio, AA and docosahexaenoic acid (DHA) had significant correlations with specific fecal flora, such as *Bifidobacterium*, *Bacteroides*, *Lactobacillus*, *Enterococcus*, *Enterobacteriaceae,* and *Candida* (Figure 1). These correlations differed among the three groups suggesting that chronic liver disease itself modifies fatty acid metabolism induced by intestinal flora. These data indicate that the relationship between gut microbiota and host metabolism differs by metabolic activity of the liver, indicating that individual "the gut-microbiota-liver network" exists in each clinical disease entities and the importance to evaluate in future studies[11].

With regards to polyunsaturated fatty acids, Wall *et al*[98] performed a mouse study investigating the effects of *Bifidobacterium breve* NCIMB 702258 administration with coadministration of α-linolenic acid on fatty acid composition of the liver, adipose tissues, large intestine and brain, and showed increased c9, t11 conjugated linoleic acid and EPA levels in the liver, while *Bifidobacterium* administration alone did not change the EPA levels in normal mice. Wall *et al*[99] also demonstrated increased EPA levels in adipose tissues from severe combined immunodeficient mice after *Bifidobacterium breve* NCIMB 702258 administration. Conjugated linoleic acid is a microbial metabolite associated with the alleviation of NAFLD[100]. Kankaanpää *et al*[101] reported the effects of 8 wk of *Bifidobacterium* Bb-12- or *Lactobacillus* CG-supplemented infant formula administration on the plasma fatty acid composition in infants. They found that *Bifidobacterium* decreased serum phospholipid EPA to 61% and AA levels to 77% compared with baseline values. In addition, *Lactobacillus* decreased EPA to 22% and AA to 62%. These reports described the effects of probiotics on host fatty acid compositions, but the results differed among the probiotics used and the host conditions. It was recently demonstrated that exposure of the human intestinal mucosa to *Lactobacillus plantarum* WCFSI induced the upregulation of genes in the intestinal mucosa involved in lipid/fatty acid transport, uptake and metabolism, such as CD36 and microsomal triglyceride transfer protein[102]. Several genes participating in mitochondrial and peroxisomal fatty acid metabolism were also upregulated[102].

With regards to liver damage and metabolism in hepatitis C virus patients, hepatitis C virus genotype 3 infection perturbed glucose homeostasis through several direct and indirect mechanisms, leading to both hepatic and extrahepatic insulin resistance and accelerated disease progression including the development of hepatocellular carcinoma and type 2 diabetes[103]. Furthermore, changes in polyunsaturated fatty acids and lipid metabolism induced by hepatitis C core protein is thought to be involved in the pathogenesis of lipid metabolism disorders[102,104]. The administration of AA or EPA modulated the hepatitis C viral mechanism in hepatocytes[105]. Every step of the hepatitis C virus life cycle is intimately connected to lipid metabolism[106].

Bajaj *et al*[69] reported gut microbiota and serum/urine metabolome in a phase I randomized clinical trial using probiotic *Lactobacillus* GG (LGG) in patients with cirrhosis. They showed the safety and tolerance of 4 wk LGG administration in cirrhosis patients, which improved endotoxemia and gut dysbiosis. Furthermore, significant gut microbe-metabolome linkage was obtained by LGG as the results of system biology analysis. Figure 2 shows the correlation network among changed gut microbiota by LGG and metabolomic analysis. For example, a reduction in *Enterobacteriaceae* was associated with a linked change with anti-inflammatory cytokine IL-13 and ammoniagenic amino acids that was not seen in the placebo group. Changes in the levels of several vitamins in the blood were also observed following the co-administration of multivitamins with sufficient nutrition in both the LGG and placebo groups in their study.

Obesity-associated hepatocellular carcinoma was recently attributed to molecular mechanisms such as chronic inflammation caused by adipose tissue remodeling and pro-inflammatory adipokine secretion, ectopic lipid accumulation and lipotoxicity, altered gut microbiota, and disrupted senescence in stellate cells, as well as insulin resistance leading to increased levels of insulin and insulin-like growth factors. LPS, a pathogen-associated molecular pattern recognized by TLR4, initiated various inflammatory and oncogenic pathways to develop hepatocarcinogenesis and was enriched in the intestine of obese humans and rodents[97,107].

The complexity of the gut microbiota could be revealed using a recent systems biology culturomics-based method, genomic- and metagenomic-based methods, and proteomic- and metabolomic-based

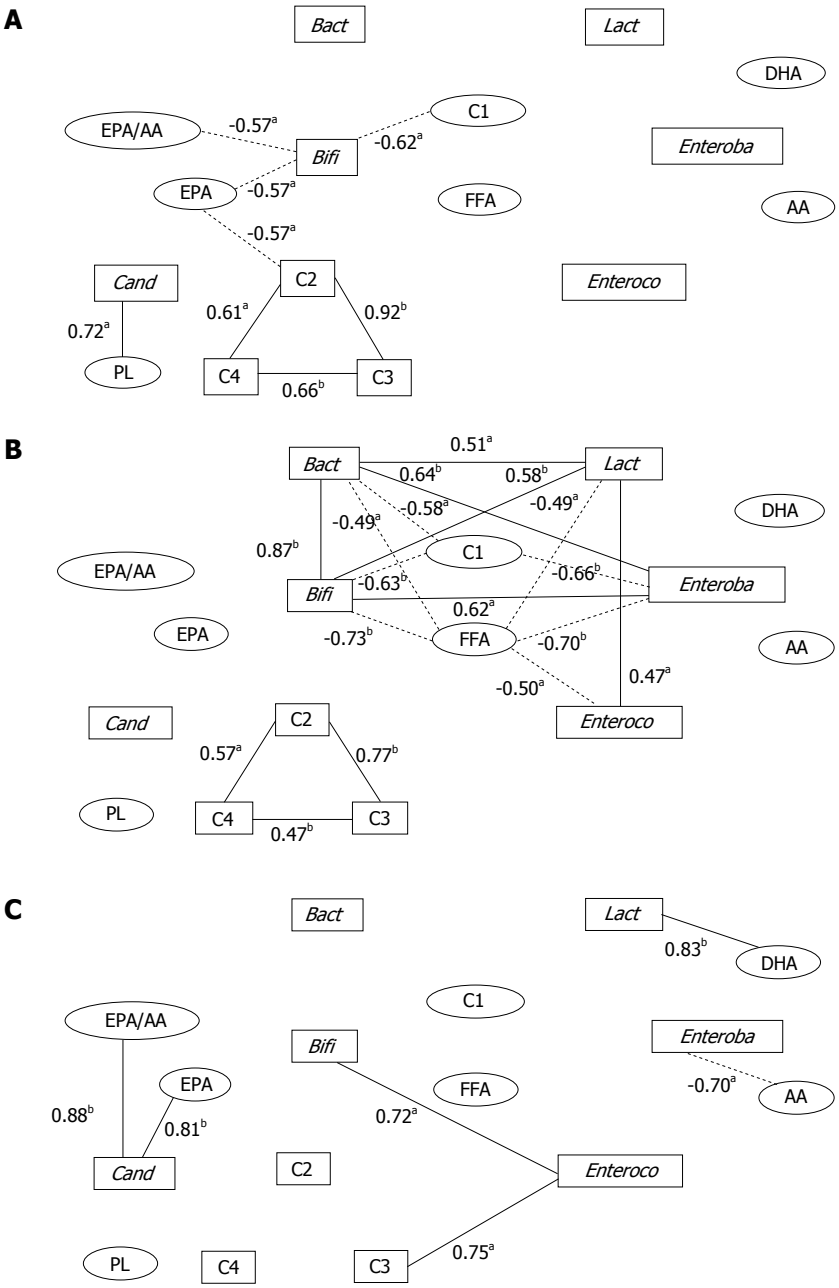

Figure 1 Correlation networks among fecal microflora and organic acid and serum organic and fatty acid concentrations in hepatic cancer patients. Square boxes indicate fecal components and ellipsoids indicate serum components. Solid lines indicate positive correlations and dotted lines indicate negative correlations. A: Normal liver group; B: Chronic hepatitis or liver fibrosis group; C: Liver cirrhosis group. $^{a}P < 0.05$ and $^{b}P < 0.01$ by Pearson's correlation coefficient test. *Bact*: *Bacteroidaceae*; *Bifi*: *Bifidobacterium*; *Lact*: *Lactobacillus*; *Enteroba*: *Enterobacteriaceae*; *Enteroco*: *Enterococcus*; *Cand*: *Candida*; C1: Formic acid; C2: Acetic acid; C3: Propionic acid; C4: Butyric acid; AA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; FFA: Free fatty acid; PL: Phospholipid. Data adapted from Usami *et al*[46].

methods[11]. Samples from the gut or other microbiota (*e.g.,* feces and saliva) are assayed on solid media selective for axenic cultivation. Isolated microbial colonies are subjected to peptide extraction before matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-based mass-spectrometry processing and species identification by peptide fingerprinting in the culturomics-based method. After standardized DNA extraction and quality control protocols, metagenomic sequences from the microbiota are generated by prosequencing selected 16S rRNA regions from microbial genomes by metagenomic-based methods. The detection of metabolites from samples such as feces, urine, blood, plasma and saliva, can be performed using metabolomic approaches including gas-chromatography mass spectrometry, proton nuclear magnetic spectroscopy (^{1}H-NMR) and a liquid chromatography mass spectrometry in metabolomic-based method. These are recommended as platforms to understand further the gut-microbiota-liver metabolic network[11,108].

This review highlighted recent studies that re-

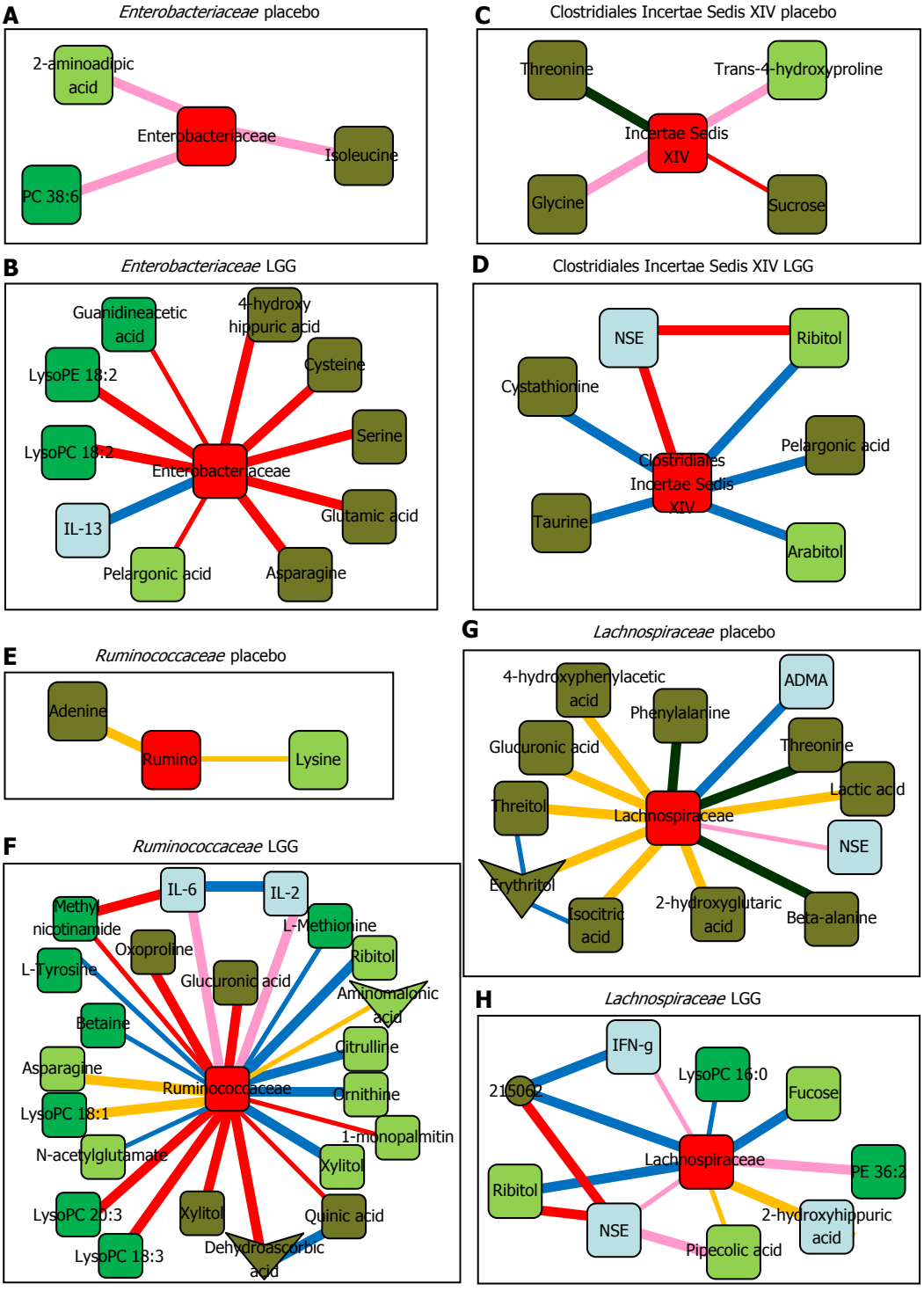

Figure 2 Sub-networks showing correlation network differences from baseline to week 8 in placebo and in Lactobacillus GG groups separately centered on selected bacterial taxa. Color of nodes: Blue, inflammatory cytokine; light green, serum metabolites; dark green, urine metabolites. Color of edges: pink, negative remained negative but there is a net loss of negative correlation; dark blue, negative changed to positive; yellow, positive remained positive but there is a net loss of positive correlation; red, positive to negative; dark green, complete shift of negative to positive; military green, complete shift of positive to negative. A, B: Sub-networks of correlation changes centered around *Enterobacteriaceae*; C, D: Sub-networks of correlation changes centered around Clostridiales Incertae Sedis XIV; E, F: Sub-networks of correlation changes centered around *Ruminococcaceae*; G, H: Sub-networks of correlation changes centered around Lachnospiraceae. NSE: Neuron-specific enolase; IL: Interleukin; IFN: Interferon; LysoPC: Lysophosphatidylcholine; LysoPE: Lysophosphatidylethanolamine; ADMA: Asymmetricdimethylarginine; Rumino: *Ruminococcaceae*. Data adapted from Bajaj *et al*[69].

ported an association between gut microbiota and host metabolism in cirrhosis. However, those reports mark the beginning of a new research area of the gut-liver axis. The liver is the central organ in host-metabolism and future studies are important and will form a new research area in the setting of the gut-microbiota-liver metabolic network. Hopefully this will contribute to interventions for the development of

liver cirrhosis and related infectious and non-infectious complications including metabolic disturbances evoked by the gut-liver axis, especially in ALD, NAFLD and hepatocarcinogenesis.

CONCLUSION

Gut microbiota can produce a diverse range of compounds that play a major role in regulating the activity of distal organs and the liver is strategically positioned downstream of the gut. We are gaining increased insight into the close relationship between the gut and the liver evoked by systemic metabolic changes. The evaluation of the gut-microbiota-liver metabolic network and the intervention of these relationships using probiotics, synbiotics, prebiotics with sufficient nutrition might aid the development of treatment and prevention for liver cirrhosis patients.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Towards curative therapy in gastric cancer: Faraway, so close!

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Abstract

Although recent diagnostic and therapeutic advances have substantially improved the survival of patients with gastric cancer (GC), the overall prognosis is still poor. Surgery is the only curative treatment and should be performed in experienced centers. Due to high relapse following surgery, complementary and systemic treatment aimed at eradicating micrometastasis should be performed in most cases. Cytotoxic treatments are effective in downstaging locally advanced cancer, but different sensitivities and toxicities probably exist in different GC subtypes. Current treatment protocols are based primarily on clinical data and histological features, but molecular biomarkers that would allow for the prediction of treatment responses are urgently needed. Understanding how host factors are responsible for inter-individual variability of drug response or toxicity will also contribute to the development of more effective and less toxic treatments.

Key words: Gastric cancer; Multidisciplinary treatment; Therapeutic strategies; Curative surgery

Core tip: There has been much progress in the past decades regarding the identification of risk factors for gastric cancer and understanding its pathogenesis. Diagnostic and therapeutic management of this disease has also improved significantly in the past few years. Despite these advances, prognosis remains dismal, and new therapeutic options are urgently needed. Hopefully, in the years to come, treatments will be tailored for a given patient based on tumor characteristics and host factors, with the aim of increasing therapeutic efficacy and decreasing toxicity. Faraway, so close!

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INTRODUCTION

Gastric cancer (GC) is a major public health issue, and it is the fourth most common cancer and the second leading cause of cancer-related death[1]. It is usually diagnosed at an advanced stage, and, consequently, the prognosis is dismal. Although surgery is the definitive therapy, rates of recurrence are high, creating the need for neoadjuvant or adjuvant therapy. These therapies have improved significantly the 5 year survival of these patients but not all patients benefit equally from these treatment options. The ability to predict patient response to specific therapies would be particularly valuable and would allow for the stratification of patients for personalized treatment strategies, likely with less toxicity. Recent advances have improved our understanding of gastric carcinogenesis with an unprecedented opportunity of developing novel therapeutic strategies. Exploring and validating tissue-based biomarkers are ongoing processes, which will certainly open new avenues for treating and improving the prognosis of patients with GC.

RISK FACTORS FOR GC

Like other human cancers, GC is the end result of the interplay of environmental and susceptibility factors. The striking geographic variation in GC incidence reflects early role of environmental exposure rather than genetics, as migration studies have confirmed a decline in incidence in subsequent generations. The only environmental factor that is considered to be a type I carcinogen by the World Health Organization is *Helicobacter pylori* (*H. pylori*)[2]. This bacterium can have a lifelong uneventful relation with its host but, in a minority of cases, causes peptic ulcer, both intestinal and diffuse type gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphoma. About 50% of the world's population is infected with *H. pylori*, but less than 0.5% of infected individuals will develop GC. This disparity reflects the variation in the pathogenicity of bacterial strains as well as host inflammatory genetic susceptibility factors such as interleukin (IL)-1B, IL-8, IL-10, interferon (IFN)-gamma, and tumor necrosis factor beta (TNF-β) polymorphisms[2]. *H. pylori* infection causes chronic inflammation, accumulation of reactive oxygen species (ROS), and oxidative damage in the gastric mucosa, thereby promoting the sequential progression of normal gastric epithelium through atrophic gastritis, intestinal metaplasia, and dysplasia to carcinoma. Advanced atrophic corpus-predominant gastritis and subsequent development of intestinal metaplasia provide the histological base for GC genesis[3]. This model of precancerous lesions is currently accepted, and surveillance recommendations apply to patients at increased risk[3]. The intestinal-type GCs are more related to atrophic gastritis, intestinal metaplasia, and dysplasia, but *H. pylori* infection is also associated with an increased risk of diffuse-type GC.

In addition to *H. pylori*, dietary and lifestyle factors may also modify the risk of developing GC. Low socioeconomic status and associated conditions have been linked with a two-fold increase in GC risk[4]. Subjects belonging to a low socioeconomic status have a higher prevalence of *H. pylori* infection, more frequent smoking habit, and less vegetables and fruit intake than the general population[5]. In an analysis of the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST), there was a 45% higher risk of GC associated with ever smoking compared to never smoking[6]. In a meta-analysis with 42 articles, Ladeiras-Lopes *et al*[7] concluded that smoking is the most important behavioral risk factor for GC. Heavy alcohol intake has been linked to some increased GC risk, mainly in men[8]. Nonetheless, as heavy drinkers usually smoke and have a poor diet, there may be some confounding factors in these conclusions[4]. Among dietary factors, N-nitroso compounds (including nitrosamine) are proven animal carcinogens. Potential sources of N-nitroso compounds are beer, processed (smoked, cured, salted, and pre- served) meats, red meat, and tobacco smoke[8]. In the EPIC cohort, the authors found no association between nitrites and nitrosodimethylamine intake and GC risk, but endogenous production of N-nitroso compounds was significantly associated with non-cardia cancer risk[9].

A meta-analysis in 2012, including 2076498 patients, showed a significant positive association between high salt intake and GC[10]. High salt intake damaged gastric mucosa and increased the susceptibility to carcinogenesis in studies with rodents.

In respect to protective factors, intake of non-starchy vegetables and fruits has been associated with a moderately decreased risk of GC in many cohort-, population- and hospital-based case-control studies[4,5]. In a reanalysis of the EPIC cohort, a negative and significant association was observed between total vegetable, fruit, and flavonoid intake and dietary total antioxidant capacity and risk of GC[11-13]. This protection afforded by vegetables and fruits may derive from their content in antioxidants (such as vitamin C), which may reduce the formation of N-nitroso compounds in the stomach[5].

A recent large European prospective cohort study investigated the combined impact of the above-cited behaviors on GC risk using a healthy lifestyle index[14]. The authors concluded that adopting a combination of lifestyle behaviors, including not smoking, limiting alcohol consumption, following a healthy dietary

pattern (represented by the Mediterranean diet), and having a normal body mass index can dramatically decrease the burden of GC. In 2003, in a meta-analysis by Wang *et al*[15] including 2831 GC patients, regular NSAIDs users had a reduced risk of GC (OR = 0.78, 95%CI: 0.69-0.87). These results have recently been confirmed in a wide systematic review[16]. The pro-apoptotic and anti-angiogenesis effects of NSAIDs are known to inhibit carcinogenesis in patients with colonic polyps, and NSAIDs may act in a similar way in gastric mucosa[4]. To date, no randomized controlled studies exist on the long-term effect of NSAIDs alone on the development of GC, and the alleged protective effect could simply reflect the "protective user effect", as most individuals eligible for sustained NSAID chemoprophylaxis do not usually suffer from gastric disease.

The decrease of distal GC prevalence that has been consistently described in a number of studies[17] could very well be the result of life style changes associated with improvement of economic status, better hygiene, and consequent decrease of *H. pylori* infection[4,5].

PATHOGENETIC MECHANISMS OF GASTRIC CARCINOGENESIS

About 95% of gastric tumors are adenocarcinomas, which can be classified into well differentiated (intestinal), undifferentiated (diffuse), and mixed types. Further knowledge about GC heterogeneity has been provided by The Cancer Genome Atlas Research Network. Through the molecular characterization of 295 gastric adenocarcinomas, four GC subtypes have been proposed: microsatellite unstable tumors; genomically stable tumors; tumors displaying chromosomal instability; and Epstein-Barr positive tumors[18]. Hopefully, this subtype analysis will aid in the development of tailored therapeutic strategies for selected patients.

Like other cancers, GC is a complex, multistep, and molecularly heterogeneous disease, involving deregulation of canonical oncogenic pathways, such as p53, Wnt/β-catenin, and nuclear factor (NF)-κB, among others. While most intestinal-type of GCs progress through the multistep cellular dedifferentiation described by Correa[19], most diffuse-type cancers involve the sporadic or syndromic loss of expression of adhesion protein E-cadherin (CDH1). This is a calcium dependent cell-to-cell adhesion glycoprotein that plays a critical role in maintaining the normal epithelium architecture. The cytoplasmic domain of this molecule interacts with β-catenin, forming strong cohesive nets between the actin cytoskeleton, essential for processes of cell-cell adhesion. Inactivation of CDH1 by mutation, deletion, or aberrant methylation leads to enhanced cellular motility resulting in tumor dedifferentiation and invasiveness[20]. Inactivation of CDH1 has been described in over 50% of diffuse GC but also in a small proportion of intestinal-type tumors[20].

Stem cell hypothesis

Most of the molecular events described above have been extensively characterized. Irrespective of the type or order in which these events to promote gastric carcinogenesis, the stem cell hypothesis states that tumors are heterogeneous, and there is a subset of cells capable of self-renewal, asymmetrical division, and differentiation with the ability of generating a new tumor. Takaishi *et al*[21] identified CD44 as a gastric stem cell marker. The origin of cancer stem cells (CSCs) could be differentiation of epithelial stem cells (epithelial-mesenchymal transition (EMT)) or recruitment of bone marrow derived stem cells (BMDSCs). Houghton *et al*[22] published a breakthrough paper in *Science* in 2004, claiming that GC could originate in the bone marrow. Using a model of *Helicobacter* infected mice, the authors demonstrated that BMDSCs repopulated the gastric epithelium and progressed from metaplasia to dysplasia and later to intra-epithelial cancer. The authors proposed that chronic inflammation induced by *H. pylori* promoted cytokine release and mesenchymal stem cell recruitment from the bone marrow. These bone marrow stem cells are capable of homing to the stomach epithelium and differentiating into gastric cells through fusion[23].

One of the most important steps in carcinogenesis is the moment when cancer becomes a systemic disease. The EMT is the developmental process whereby epithelial cells acquire the migratory capacities of mesenchymal cells. These mechanisms involve replacement of E-cadherin by N-cadherin, metalloproteinase increase, and transcription of Snail and ZEB[24]. Very recently, Choi *et al*[25] showed that *H. pylori* induced EMT by comparing the expression of TGF-B1 and EMT markers (Twist, Snail, Slug, vimentin, and E-cadherin) in controls and patients with gastric dysplasia and early GC (EGC) before and after *H. pylori* eradication with a follow up of 46 mo. TGF-B1, Twist, Snail, Slug, vimentin, and CD44 were upregulated in patients with dysplasia and EGC while E-cadherin was decreased. After *H. pylori* eradication, E-cadherin expression was enhanced while the other markers were reduced. These authors proposed that *H. pylori* triggers both the EMT pathway and the emergence of gastric stem cells.

As appealing and out of the box as the stem cell hypothesis may be, it has not yet produced specific therapeutic targets, and its mechanisms seem too ubiquitous to be targeted.

Although much has been made in the past decades regarding the understanding and identification of genetic and epigenetic events that can drive normal gastric mucosa to cancer, we now need to use proteomic and metabolic approaches to design targeted and effective therapies in patients with GC.

Table 1 Randomized trials of surgery with and without neoadjuvant or perioperative chemotherapy in resected gastric cancer

Ref.	*n*	Chemotherapy	Hazard ratio for survival (95%CI)
Cunningham *et al*[27], 2006	503	ECF	0.75 (0.60-0.93)
Ychou *et al*[49], 2011	224	PF	0.69 (0.50-0.95)
Schuhmacher *et al*[28], 2010	144	PF	No significant survival difference

ECF: Epirubicin/cisplatin/5-fluorouracil; PF: Cisplatin/5-fluorouracil.

Given the role of these molecular events in directing the pathogenesis of GC, studying their signatures and developing them as biomarkers for targeted therapies is likely to impact significantly the outcome of these patients

THERAPEUTIC STRATEGIES IN GC

Perioperative therapies - chemo and chemoradiotherapy

Currently, surgical resection is the only curative therapy for non-metastatic gastric adenocarcinoma. However, since GC may be a systemic disease from the beginning, it follows that patients submitted to surgery alone were prone to locoregional or distant recurrences of their disease.

Due to large scale randomized trials demonstrating that preoperative and perioperative chemotherapy (CT) improves the clinical outcome for patients with GC[26-28], a standard medical treatment of GC has been defined[29]. Patients with potentially resectable tumors are treated with surgery and perioperative CT or postoperative chemoradiotherapy (CRT)[30,31]. In most European countries, combined preoperative and postoperative administration of CT, as in the multinational MAGIC trial[27], is the preferred treatment strategy. In North America, most centers perform postoperative CRT according to the large American Intergroup trial (INT0116). The latter is criticized by some, as inadequate surgical lymphadenectomy may have led to overestimation of the benefit[31]. This is supported by retrospective data from the Dutch D1D2 trial, which demonstrated that CRT reduced local recurrence rates following D1 resection but provided no benefit in patients who have undergone D2 resection[32].

Cytotoxic therapy provides positive response rates ranging from 20%-60%[33], which is a major breakthrough if we remember that two decades ago CT was used solely in the palliative setting because the chemosensitivity of GC was considered very mild.

Although there are a few studies evaluating clinical and pathological predictors of response and prognostic factors in the neoadjuvant setting, none of the potential markers have been validated in prospective studies[34-36].

Neoadjuvant/ perioperative chemotherapy

Neoadjuvant CT is administered as a means of "down-staging" a locally advanced tumor prior to an attempt at curative resection. This approach has been applied to patients thought to have resectable disease as well as those with apparently unresectable but nonmetastatic disease. One proposed advantage is better compliance to CT, usually, in the neoadjuvant setting. Another benefit of neoadjuvant CT is in patients who are at high risk of developing distant metastases (*e.g.*, those with bulky T3/T4 tumors, visible perigastric nodes) who may be spared the morbidity of unnecessary gastrectomy if evidence of distant metastases emerges after CT.

Three large, adequately powered trials have directly compared surgery with or without neoadjuvant or perioperative CT, two of which demonstrated a survival benefit for this approach[26-28]. A meta-analysis of these three trials plus two other trials, which compared preoperative oral fluoropyrimidine *vs* surgery alone[37,38], and seven other smaller trials, which compared a variety of preoperative CT regimens *vs* surgery alone, concluded that neoadjuvant CT was associated with a statistically significant benefit in terms of both overall survival (OR = 1.32, 95%CI: 1.07-1.64) and progression free survival (PFS) (OR = 1.85, 95%CI: 1.39-2.46)[30]. Furthermore, neoadjuvant CT was associated with a significantly higher complete (R0) tumor resection rate (OR = 1.38, 95%CI: 1.08-1.78) and did not significantly worsen rates of operative complications, perioperative mortality, or grade 3 or 4 adverse effects (Table 1).

In terms of patient selection for this approach, it is reasonable to utilize the eligibility criteria for the MAGIC trial: patients of any age with a performance status of 0 or 1, with a histologically proven adenocarcinoma of the stomach that was considered to invade the muscular propria (T2) and/or with positive lymph nodes N+, and with no evidence of distant metastases or locally advanced inoperable disease, as evaluated by computed tomography, ultrasonography, and laparoscopy[27].

Neoadjuvant chemotherapy vs neoadjuvant chemoradiotherapy

Preoperative combined CRT and radiation therapy (RT) is more commonly used for esophageal, esophagogastric junction (EGJ), and gastric cardia cancers than for potentially resectable non-cardia gastric adenocarcinomas. Neoadjuvant CRT was compared with induction CT alone in the multicenter German POET[39]. Although there were potentially clinically meaningful survival differences that favored CRT, they were not statistically significant. Furthermore, whether the results can be extrapolated to patients with true non-cardia GC is uncertain. The ongoing TOPGEAR trial addresses the question whether neoadjuvant CRT is superior to CT in a phase Ⅱ/Ⅲ setting[40].

Table 2 Randomized trials of adjuvant chemo or chemoradiotherapy in resected gastric cancer

Ref.	*n*	Regimen	3 yr Disease free survival rate (%; *P* value)
Lee *et al*[41], 2012	458	XP *vs* XP/XRT/XP	No significant Disease free survival difference
Yu *et al*[45], 2012	68	5FU/LV *vs* 5FU /LV/RT	44.1 *vs* 67.7 ($P < 0.05$)
Kim *et al*[44], 2012	90	5FU/LV *vs* 5FU/LV/RT	No significant Disease free survival difference
Kwon *et al*[46], 2010	61	5FU/LV *vs* 5FU/LV/RT	No significant Disease free survival difference
Bamias *et al*[43], 2010	147	DP *vs* DP/RT	No significant Disease free survival difference

X: Capecitabine; 5-FU: 5-fluorouracil; LV: Leucovorin; P: Cisplatin; D: docetaxel; RT: Radiotherapy (45 Gy).

Adjuvant chemoradiotherapy vs adjuvant chemotherapy

Adjuvant CT has been directly compared with adjuvant CRT in several trials[41-46], only one of which has shown a significant overall survival benefit for the addition of RT to CT[41]. The largest trial, the ARTIST trial, compared CT alone with the addition of RT to cisplatin plus capecitabine (XP). CRT did not significantly reduce recurrence rates, although in a post-hoc subgroup analysis, patients with nodal metastases had superior disease-free survival with combined therapy compared with CT alone[41]. In the latest update, at a median follow-up of 84 mo, 3 year disease-free survival (the primary endpoint) was not significantly better in patients who received combined modality therapy[42]. The hypothesis that adjuvant CRT may represent a better approach than adjuvant CT for patients with node-positive disease will be tested in a successor trial, the ARTIST-Ⅱ trial.

The only trial to show a significant survival benefit for the addition of RT, randomly assigned 68 patients undergoing complete resection with a D1 or D2 lymph node dissection. The 3 year disease-free survival rate was significantly higher in the CT group (56% *vs* 29%), as was overall survival (68% *vs* 44%)[45].

Although studies are still ongoing, the available data does favor the addition of adjuvant radiotherapy in the treatment of GC (Table 2).

When adjuvant therapy is used, the optimal regimen has not been established. Acceptable alternatives include epirubicin, cisplatin, and infusional 5-fluorouracil (ECF), as was used in the perioperative MAGIC trial[27]. Results with adjuvant capecitabine plus oxaliplatin (CAPOX, XELOX), as was used in the CLASSIC trial[47]; or XP, as was used in the ARTIST trial[41], are not as advanced as those of perioperative ECF (as was used in the MAGIC trial) or S-1[48].

The optimal time between surgery and postoperative treatment varies widely. In the MAGIC trial[27], it was initiated 6 to 12 wk after surgery, in the Intergroup trial (INT0116)[31], it was between 4 to 7 wk, and in ACTS-GC[48] patients, it started within 6 wk after surgery.

Regarding compliance to treatment, MAGIC[27] and FCCNLC[49] trials reported that postoperative treatment was concluded in only 42% to 50% of the patients, demonstrating the importance of preoperative CT and questioning the use of postoperative treatment in the perioperative setting.

In conclusion, the optimal way to integrate combined modality therapy has not been definitively established. Decisions are often made based on institutional and/or patient preference. As science moves increasingly toward molecular targeted therapy, biologic agents hold great promise in the treatment of this disease as well.

CURATIVE SURGERY IN GC

Optimal type of gastrectomy and the length of proximal resection margin

It is of paramount importance to discuss surgical treatment of GC, given its central role in the overall management of the disease.

Total gastrectomy (TG) is the recommended therapy for more proximal tumors in order to guarantee an appropriate proximal resection margin (PRM). For distally located tumors, subtotal gastrectomy (SG) was recommended with a PRM of more than 2-3 cm for early GC and 5-6 cm for advanced GC. In patients with poorly differentiated diffuse cancer, infiltration of the proximal resection margin by microscopic tumor deposits was a major concern, and TG was classically recommended. However, a randomized controlled trial (RCT)[50,51] assessed the incidence of microscopic resection margin involvement in patients with diffuse type GC and found no statistically significant difference between total and SG and no effect on survival. Furthermore, the authors claim that SG is associated with better nutritional status and quality of life as compared to TG. There is no total agreement regarding what should be considered an appropriate PRM in SG. As shown on Table 3, distances recommended by the German Society differ from those proposed by the Japanese Gastric Cancer Association (JGCA).

Nonetheless, if one considers SG in patients with distally located diffuse-type GC, a wider excision with intraoperative frozen section (IFS) of the resection margin is recommended[52] because they are more likely to have a positive margin. On multivariate analysis, higher T stage, higher N stage, larger tumor size, and diffuse histologic type were significant independent predictive factors for a positive margin[52-54]. Studies have shown that if PRM is confirmed to be negative for malignancy but shorter than the recommended length, further resection for a larger PRM is unnecessary, since the length of PRM has

Table 3 Criteria for adequate surgical margins

	German S3	JGCA
Resection margins	Oral, aboral circumferential	Proximal
Proximal resection margins	5 cm (intestinal type) 8 cm (difuse type)	-cT1: 2 cm -cT2-T4: 3 cm (expansive) 5 cm (infiltrative)

no prognostic impact as long as resection margin is free of tumor[55].

When PRM is positive, the benefits of reoperation always have to be balanced against the risks of this technically demanding procedure. Redo surgery appears to have the most obvious survival advantage in early stage patients, especially when few nodes are involved (N0 or N1)[56,57]. In contrast, advanced N stage patients with positive margins may not benefit from an extended re-excision. Multidisciplinary options, including CT and radiotherapy, are probably more appropriate treatments for positive-margin patients, especially in patients with bulky node disease[56,58]. This is further supported by a retrospective comparison of the Dutch D1D2 trial, where the authors observed significant improvement in survival and local recurrence rates with the use of CRT after a microscopically incomplete R1 resection[56,58].

Lymphadenectomy in resectable GC

The extent of lymphadenectomy in the treatment of GC has been debated for more than two decades. The majority of Japanese and Korean (*i.e.*, Eastern) surgeons would agree that an extended lymphadenectomy (D2) leads to improved outcomes and survival. Several large retrospective studies from those groups have illustrated an impressive overall survival that was unfortunately not reproduced in most Western series.

Early published studies in the West did not show any advantage in long-term survival of D2 lymphadenectomy as compared to D1 dissection, mainly due to an elevated morbidity and mortality associated with D2 procedure[59-62].

As shown in Table 4, only a Taiwanese study[63,64] found a significant survival advantage of D2 with respect to D1, while the British[60,65], Dutch[59,66] and Italian[67,68] trials did not find a significant difference in long-term survival comparing the two procedures. The Japanese trial[69] did not find any survival advantage of prophylactic para-aortic nodal dissection (PAND).

In contrast Roviello *et al*[70] showed that D2 dissection was performed with acceptable mortality and morbidity (2% and 17%, respectively) and Siewert *et al*[71] found improved survival for stage Ⅱ patients that underwent D2 lymphadenectomy with no increased morbidity.

More recently, the Dutch GC Group Trial[32] showed that, compared with D1, D2 lymphadenectomy was associated with lower local recurrence and lower cancer-related death rates, despite a significantly higher postoperative mortality, morbidity, and reoperation rates. The Italian GC Study Group[67] randomized 267 patients and compared the short-term results of D1 and D2 lymphadenectomy for curable GC. Pancreaticosplenectomy was not considered as a routine part of the D2 gastrectomy. This study did not show significant differences in operative mortality, morbidity, and duration of postoperative hospital stay. The authors concluded that modified D2 lymphadenectomy, a spleen-preserving D2 resection technique currently available in high-volume centers, is a safe option to treat GC of Western patients.

In order to achieve better surgical outcomes, Northern European countries carried out a centralization and standardization of surgical procedures in GC. In Denmark, this process improved short term results, where 30 d hospital mortality decreased from 8.2% to 2.4%, and the proportion of patients with at least 15 lymph nodes removed was increased from 19% to 76%[72]. Centralization of GC surgery and/or audits for GC are currently implemented in the United Kingdom, Sweden, Finland, and the Netherlands[73,74].

In conclusion, the current consensus is that for medically fit patients D2 lymphadenectomy should be the standard procedure. It should be carried out in specialized, high-volume centers with appropriate surgical expertise and postoperative care[75,76]. The German, British, and ESMO-ESSO-ESTRO guidelines adopted this as the standard of care for surgical treatment with curative intent[77].

Is there a place for laparoscopic gastrectomy?

Laparoscopic gastrectomy in GC is gaining popularity worldwide as a minimally invasive alternative treatment to traditional open surgery.

Laparoscopic surgery has the potential benefits of a decreased operative morbidity and reduced recovery times but with longer operative time.

Most meta-analyses support these benefits in distal gastrectomy, however, the oncological and long-term outcomes still need to be validated[75,76]. Postoperative morbidity is greater, particularly in total gastrectomy. According to the JGCA guidelines, D2 dissection of stations 12a or 10 can be technically demanding due to the risks of organ injury, bleeding, and/or bile and pancreatic leakage. There is also no consensus on the technique of anastomosis following a laparoscopic total gastrectomy. The introduction of a circular stapler with transorally inserted anvil has enabled esophagojejunostomy anastomosis. This procedure resembles the conventional approach by laparotomy[78].

The most common technique is laparoscopic assisted distal gastrectomy (LADG) without hand assistance, which is also the most frequently reported procedure in the current literature[79-81]. Trials are

Table 4 Selected randomized controlled trials studying the extent of the lymph node dissection for patients with gastric cancer

Ref.	Year published	Region	Extent of Lymph node dissection	Patients (*n*)	Morbidity	Mortality	5 yr overall survival (%)
Dent *et al*[62]	1988	South Africa	D1	22	22%	0%	N/A
			D2	21	43%	0%	N/A
BonenKamp *et al*[59,66]	1995	The Netherlands	D1	380	25%	4%	45
			D2	331	43%	10%	47
Cuschieri *et al*[60,65]	1996	Europe	D1	200	28%	6.5%	35
			D2	200	46%	13%	33
Wu *et al*[63,64]	2004	Taiwan	D1	110	7.3%	0%	53.6
			D3	111	17.1%	0%	59.5
Sasako *et al*[69]	2008	Japan	D2	263	20.9%	0.8%	69.2
			D2 + PAND	260	28.1%	0.8%	70.3
Degiuli *et al*[67,68]	2010	Italy	D1	133	12%	3%	66.5
			D2	134	17.9%	2.2%	64.2

PAND: Para-aortic node dissection.

currently ongoing in Japan (JCOG-0912), South Korea (KLASS and KLASS-02), and China to compare open and laparoscopic surgery in EGC[81,82]. These should provide further insight into the role of laparoscopic approach before moving to the laparoscopic treatment of locally advanced GC, especially when a TG with D2 lymphadenectomy is recommended. As such, and at the time of writing this paper, one cannot advise laparoscopic gastrectomy for treating GC, outside a clinical trial.

TREATING GC IN THE 21st CENTURY: ARE WE READY FOR PERSONALIZED THERAPY?

This is certainly an active topic of clinical and basic research not only because GC is a highly prevalent disease, but also because the treatments used may be effective but sometimes very toxic[83]. Although the prognosis and 5 year survival is still poor for patients with locally advanced GC, considerable progress has been achieved in the past two decades[84]. Besides staging procedures, which allow for a more accurate staging of the disease and enable a more appropriate selection of patients for pre-operative cytoreductive CT, both surgical and medical therapies have evolved substantially. From a surgical point of view, a modified D2 lymphadenectomy is now the standard procedure for medically fit patients with locally advanced GC in most European Centers. Short and long term results improved substantially in Western studies, as long as surgery was carried out in specialized, high-volume centers with appropriate surgical expertise and postoperative care. This was certainly a major step towards curative therapy in GC patients in the Western world.

Peri-operative CT using ECF, as in the MAGIC trial, is now the standard of care for stages Ⅱ and Ⅲ disease as recommended by ESMO-ESSO-ESTRO clinical practice guidelines[77]. This was also a major breakthrough, as up to one decade ago, CT was not systematically considered part of the curative treatment of GC.

Host factors responsible for heterogeneity of response

Although, peri-operative CT followed by radical surgery is now the standard of care for most patients with stage Ⅱ-Ⅲ non-metastatic GC, less than 50% of patients complete the full protocol due to its toxicity[27]. In this respect, there has been recent interest in exploring the relationship between body composition, especially proportions of lean and fat tissues, with treatment toxicities. The most recent definition of cancer cachexia specifically involves depletion of muscle mass, which sometimes may not impact body weight. As shown in Figure 1, patients may become sarcopenic despite a normal or even high body mass index. Muscle depletion is characterized both by a reduction in muscle size and increased proportion of inter- and intramuscular fat. Fat infiltration given by muscle attenuation (MA) is a further manifestation of the wasting process (Figure 1).

Prado *et al*[85] observed in metastatic breast cancer patients receiving capecitabine treatment that sarcopenia was a significant predictor of toxicity and time to tumor progression. The authors reported a 28-fold increase in the relative risk of grade 3 and 4 neutropenia if a patient's lean body mass (LBM) was < 89% of age and sex-adjusted norms. They hypothesized that this relationship was primarily due to a pharmacokinetic effect, as fat-free mass (LBM plus bone tissue) and total body water were better predictors of 5-FU pharmacokinetics (clearance and volume of distribution) than body surface or body weight. This has also been reproduced in patients with metastatic lung and pancreatic cancer[86]. Sarcopenia has also been associated with unfavorable clinical outcomes, such as increased length of hospital stay, increased incidence of infections for hospitalized patients, and mortality in surgical patients[87]. Lieffers *et al*[87] observed in patients aged more than 65

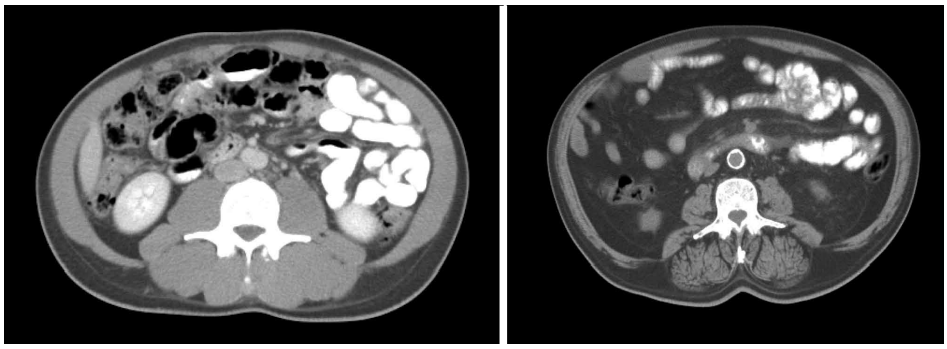

Figure 1 Axial computed tomography images of the third lumbar vertebra region from two patients with similar body mass index but different muscle and fat tissue cross sectional areas. Paraspinal muscles are clearly different between the two subjects as is mesenteric fat and fat infiltrating muscle - muscle radiation attenuation. Low relative muscularity and expanded visceral fat are associated with increased toxicity and decreased survival.

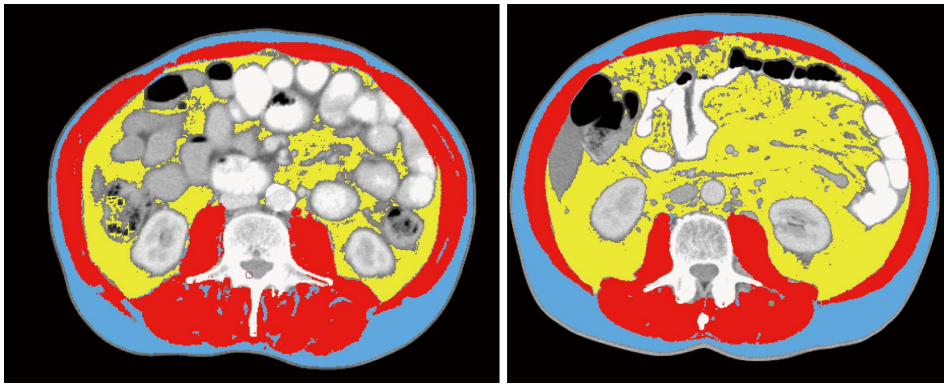

Figure 2 Lumbar computed tomography was analyzed for muscle and fat tissue cross sectional areas using an appropriate software developed by Martin *et al*[88]. Muscle mass is shown in red and were quantified within a Hounsfield unit (HU) range of -29-150, visceral fat shown in yellow, range from -150 to -50, and subcutaneous fat shown in blue, range from -190 to 30. Muscle radiation attenuation was calculated for muscle area. Although these two images might refer to two individuals with the same body mass index (23 kg/m^2) and age (73 yr), the amount of muscle mass and visceral fat, which amplifies inflammatory response, are very distinct.

years operated for colorectal cancer, sarcopenia was an independent predictor of both infection and rehabilitation care and, consequently, a longer length of hospital stay. Finally, it is important to stress that imaging of sarcopenia can be done using the CT scan performed at the time of routine imaging studies for tumor evaluation and/or restaging[88], as long as the appropriate software is available, as shown in Figure 2.

It would then be interesting to test whether these observations of body composition, muscle mass measurement, and CT toxicity also hold true in respect to patients with advanced GC selected to perioperative CT followed by radical surgery. This could shed some light on the issue why patients do not benefit equally from these treatment options.

Analysis of tumor factors that might allow for more personalized therapy

Inter-individual variability of drug response or resistance may also be related to tumor heterogeneity. The identification of predictive tumor markers at the time of diagnosis would allow for stratifying patients to more effective treatments, as current therapeutic strategies do not uniformly benefit all patients. Although very toxic in some patients, one cannot forget that complete pathologic responses are being reported with increasing frequency[89], thus making the identification of these predictive factors mandatory.

In a recent study, the authors found that pathologic complete response was observed in 20% (10/50) of patients and a further 20% (10/50) achieved near complete histological remission (< 10% residual tumor). Among these very good responders, 85% (17/20) had intestinal type tumors, 10% (2/20) had diffuse tumors, and 5% (1/20) had mixed type tumors[89].

In regard to molecular markers, and similarly to what occurs in colorectal cancer[90], MSI status seems to affect both the prognosis and the response to 5-FU based chemotherapies. One study found 5-FU based adjuvant CT prolonged disease-free survival in patients with GC stage Ⅱ and Ⅲ disease only in patients with tumors MSS or MSI-low, in contrast with the MSI-high group who did not seem to benefit from this type of therapy[91]. However, these are conflicting data, as another study did not find that MSI status significantly affected response to 5-FU CT[92].

Current research is thus focusing on identifying

cancer biomarkers, which will elucidate treatment response and drug resistance mechanisms[93]. Real progress will only be achieved through the development of new treatment options that have reduced cell toxicity compared with that of standard therapeutic regimens. Currently, except for the status of human epidermal growth factor-2, which is used to guide trastuzumab therapy, no other biomarkers are used in clinical practice.

CONCLUSION

Considering the amount of effort that has been put in clarifying the pathogenesis of GC, we are now hoping that these new discoveries will lead to the translation of these insights into the clinical arena. New proteomic technologies that promote large-scale sample screening will hopefully open new avenues for targeted and personalized therapies in patients with GC[94]. As much as unraveling gastric carcinogenesis seems closer and closer, concepts such as the migrating cancer stem cell remind us that this enigma is still faraway from being solved. Faraway, so close!

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Second-line treatment of metastatic gastric cancer: Current options and future directions

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Abstract

Gastric cancer remains one among the leading causes of cancer-related deaths, regardless of its decreasing incidence and newly available treatment options. Most patients present at an advanced stage and are treated with upfront systemic chemotherapy. Those patients receiving first-line therapy may initially respond to treatment, but many of them relapse over time. In such condition, second-line treatment for disease progression remains the only available option. Although there exists no standard approach in the second-line setting, several phase Ⅲ trials have shown modest survival benefit in patients receiving irinotecan, taxane and ramucirumab over the best supportive care or active agents. This review analyzes the currently available treatment regimens and future directions of research in the second-line setting for metastatic gastric cancer with the best available evidence. Additionally, the prognostic factors that influence patient survival in those receiving second-line therapy are discussed.

Key words: Metastatic gastric cancer; Second-line chemotherapy; Targeted therapy; Taxane; Irinotecan; Ramucirumab

Core tip: This systematic review has analyzed the currently available treatment options with chemotherapy and targeted agents in the second-line treatment of metastatic gastric cancer. In addition, this review has discussed the future directions of research and the prognostic factors that influence patient survival in those receiving second-line therapy for metastatic gastric cancer.

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INTRODUCTION

Gastric cancer (GC) remains one of the major causes of cancer-related deaths ranking at number three, despite its decreasing incidence. About one million new cases of gastric cancer were estimated to have occurred in 2012, making it currently the fifth most common malignancy in the world, behind cancers of the lung, breast, colorectum and prostate. More than 70% of diagnosed gastric cancer cases are registered in developing countries[1-3].

The disease presents as localized disease in only one-third of patients and as locally advanced or metastatic disease in the remaining two-thirds of patients. Surgery (subtotal or total gastrectomy with radical lymph node dissection) remains the primary mainstay in the treatment of GC. Despite the curative-intent resections, in up to 70% of cases, the relapse rate remains high[4]. In such patients, 5-year survival rates do not exceed 25% and prognosis remains poor[5].

Most of the patients, including those with early-stage disease, relapse at some point during the course of their disease. Among available treatment modalities, only systemic chemotherapy has demonstrated a superior survival rate in this group of patients[6]. Most patients do not respond or relapse within a short time from the end of first-line therapy. The literature shows that approximately 20%-30% of patients receive further treatment with second-line chemotherapy[7]. In the past, various cytotoxic agents (5-fluorouracil, cisplatin, mitomycin C, methotrexate, docetaxel, paclitaxel, nab-paclitaxel, pemetrexed, S-1, irinotecan and oxaliplatin) have been studied extensively either as monotherapy or in combination in the second-line setting. The median OS of patients receiving second-line therapy ranges from 3.5 to 10.7 mo, with an objective response rate of 4.8%-52.3%. There were no effective treatment options until the positive results of recent phase Ⅲ studies were published.

This systemic review evaluates the currently available evidence on the therapeutic options in the second-line setting. Additionally, the prognostic factors that influence patient survival in those receiving second-line therapy are discussed.

LITERATURE REARCH

The literature database search for second-line therapy in metastatic gastric cancer was performed using MEDLINE and PubMed for original articles published from a dataset of a minimum of 25 patients, review articles, and key abstracts from articles published in English during the period from 1990 to 2015.

First-line therapy

In metastatic gastric cancer, systemic chemotherapy has been shown to improve overall survival (OS) and quality of life (QoL) compared with best supportive care (BSC) alone[8-10]. In patients receiving first-line therapy for metastatic gastric cancer, the median survival time ranges from 9.5-13 mo with objective responses ranging from 25%-54%[11-16]. The meta-analysis performed by Wagner *et al*[17], which included 35 trials with 5726 patients, demonstrated a significant survival benefit in favor of combination chemotherapy (HR = 0.82; 95%CI: 0.74-0.90, 1914 patients) compared with single-agent chemotherapy; a significant survival benefit was observed for regimens including 5-FU, anthracyclines and cisplatin (HR = 0.82; 95%CI: 0.73-0.92, 1147 patients), and non-significant survival benefits in favor of the Irinotecan- (HR = 0.86; 95%CI: 0.73-1.02, 639 patients) and docetaxel-containing (HR = 0.93; 95%CI: 0.75-1.15, 805 participants) regimens were demonstrated. This shows that a multi-agent chemotherapy regimen comprising fluoropyrimidines and platinum derivatives acts as an effective regimen in the first-line therapy for metastatic gastric cancer. When delivering first-line therapy, one should remember that the REAL Ⅱ study established the non-inferiority of capecitabine to infusional 5-fluorouracil (HR = 0.86, 95%CI: 0.80-0.99) and the non-inferiority of oxaliplatin to cisplatin (HR = 0.92; 95%CI: 0.80-1.10) in two-by-two comparisons[13]. Additionally, Guimbaud *et al*[16] showed that FOLFIRI (irinotecan, 5-fluorouracil and leucovorin) is an acceptable alternative to a platinum-based ECX (epirubicin, cisplatin and capecitabine) regimen in first-line settings, especially in patients who are not able to receive platinum-based agents. Trastuzumab in combination with chemotherapy was found to significantly prolong survival when given as a first-line treatment for patients with HER2-positive gastric cancer[18].

Second-line therapy

Cytotoxic agents: A larger number of phase Ⅱ[19-61] and phase Ⅲ[62-78] studies have been conducted in the second-line setting, which are summarized in Tables 1, 2 and 3. Data from phase Ⅲ trials show that the median OS ranges from 3.7 to 13.9 mo, with an objective response rate of 13%-27%.

The German AIO trial[62] was the first randomized trial that studied whether second-line chemotherapy could prolong survival in gastric cancer. In this study, irinotecan was compared with BSC to show a survival benefit in patients with metastatic gastric and gastro-esophageal junction adenocarcinoma. This randomized phase Ⅲ study included 40 patients. The study was terminated prematurely due to slow patient accrual. All the patients had received prior fluoropyrimidine/platinum combination and exhibited disease progression within 6 mo following first-line therapy. The

Table 1 Phase Ⅱ studies on second-line therapy (Monotherapy) in metastatic gastric cancer

Ⅱ line agent	Total/eval pts	Performance status, *n*	Doses (mg/m^2)	Treatment ORR	Median TTP (mo)	Median PFS (mo)	Median OS (mo)	Toxicity, grade 3-4 (%)	Ref.
Irinotecan	37/35	ECOG 0/1 - 3/34	Iri - 125 d1 weekly × 4 wk, q42	20.0%	2.6	NR	5.2	A/L/N/T/N-E/D/FN/I - 56.8/45.9/67.6/8.1/ 18.9/18.9/16.2/8.1	Chun *et al*[19], 2004
Irinotecan	39/30	ECOG 0/1/2 - 18/10/2	Iri - 100 d1,d8,d15 q28	15.3%	2.9	NR	8.8	A/N/Leu/Ano/D/F/- 13/30/17/23/17/13	Mochizuki *et al*[20], 2013
Paclitaxel	36/36	ECOG 0/1/2 - 7/20/9	Ptx - 225 d1 q21	22.2%	5.0	NR	8.0	L/T - 17/17	Cascinu *et al*[21], 1998
Paclitaxel	38/25	ECOG 0/1/2 - 12/15/11	Ptx - 80 d1, 8, 15 q28	24.0%	2.1	NR	5.0	L/N/T/Nau/Ano/D/ Neu - 29/32/8/3/3/3/6	Hironaka *et al*[22], 2006
Paclitaxel	45/44	ECOG 0/1/2 - 26/13/6	Ptx - 80 d1, 8, 15 q28	16.0%	NR	2.6	7.8	A/N/L/D/N-E/As/Neu - 11/16/18/2/4/11/2	Kodera *et al*[23], 2007
Docetaxel	30/29	KPS Median 70 (R 60-100%)	Dtx - 100 d1 q21	17.0%	NR	NR	6.0	A/L/N - 7/7/18	Giuliani *et al*[24], 2003
Docetaxel	154/86	ECOG 0-1/2 - 130/24	Dtx - 75 d1 q21	14.0%	2.6	NR	7.2	A/N/FN/As - 10.5/12.5/9.9/13.6	[1]Jo *et al*[25], 2007
Docetaxel	49/49	ECOG 0/1/2 - 9/36/4	Dtx - 75 d1 q21	16.5%	2.5	NR	8.3	A/L/N/FN/B/As/D/ Neu - 5.4/4.8/5.4/5.4/1.2/ 10.8/2.4/8.4	Lee *et al*[26], 2008
Nab-Paclitaxel	56/54	ECOG 0/1 - 33/23	Nab-Ptx 250 d1 q21	27.8%	NR	2.9	9.2	A/L/N/Ly/Neu - 7.3/20/49.1/10.9/23.6	Sasaki *et al*[27], 2014
Pemetrexed	34/30	ECOG 0/1/2 - 7/20/7	Pem - 500 d1 q 21	63.6%	NR	2.3	6.4	A/N/T/As - 2.9/2.9/2.9/5.8	Zhang *et al*[28], 2015
Sunitinib	78/69	ECOG 0/1 - 26/52	Sun - 50 mg/d × 4 wk, q42	2.6%	2.3	2.3	6.8	A/L/N/T/E/D/HFS/S - 16.7/11.5/29.4/34.6/ 3.8/2.6/6.4/1.3	Bang *et al*[29], 2010
Everolimus	54/53	ECOG 0/1 - 32/21	Ev - 10 mg/d d1-28	0%	NR	2.7	10.1	P (gr 1-2)/A/Ly/As/S/ Ano/HyG/HyP/HyN/ - 15.1, 9.4/7.5/5.7/ 5.7/ 5.7/3.8/3.8/9.6	Doi *et al*[30], 2010

[1]Retrospective study. Dtx: Docetaxel; Ev: Everolimus; F: 5-fluorouracil; FA: Leucovorin; Iri: Irinotecan; MMC: MitomycinC; Nab-Ptx: Nab-paclitaxel; Pem: Pemetrexed; Ptx: Paclitaxel; Sun: Sunitinib; ECOG: Eastern co-operative oncology group; KPS: Karnoffsky performance status; NR: Not reported; ORR: Objective response rate; OS: Overall-survival; Pts: Patients; PFS: Progression-free survival; R: Range; TTP: Time-to-progression; A: Anemia; As: Asthenia; Ano: Anorexia; B: Bilirubin; D: Diarrhea; E: Emesis; FN: Febrile neutropenia; HFS: Hand-foot syndrome; HyG: Hyperglycemia; HyN: Hyponatremia; HyP: Hypophosphatemia; I: Infection; L: Leucocytopenia; Ly: Lymphopenia; M: Mucositis; N: Neutropenia; Neu: Sensory neuropathy; Nau: Nausea; N-E: Nausea and vomiting; T: Thrombocytopenia.

irinotecan arm and BSC arm had 21 and 19 patients with Eastern Cooperative Oncology Group Performance Status (ECOG PS) 0-1/2 in 17/4 and 14/5 patients, respectively. This showed that the administration of irinotecan (250 mg/m^2 d1, escalated up to 350 mg/m^2, every 3 wk) as second-line chemotherapy significantly prolongs OS when compared with BSC. This was the first evidence that second-line chemotherapy resulted in substantial improvement of survival. The median OS was 4.0 (95%CI: 3.6-7.5) mo in the irinotecan arm and 2.4 (95%CI: 1.7-4.9) mo in the BSC arm, HR = 0.48 (95%CI: 0.25-0.92), P = 0.023. There were no objective responses with 58% stable disease (SD) in the irinotecan arm. However, 44% of patients had tumor-related symptom relief. The most common grade 3-4 toxicities were diarrhea (5 patients) and febrile neutropenia (2 patients)[62].

In the Korean phase Ⅲ randomized trial[63], 202 patients with metastatic gastric cancer and an ECOG PS of 0 or 1 received one or two prior chemotherapy regimens involving both a fluoropyrimidine and a platinum agent and were randomly assigned at a ratio of 2:1 to either salvage chemotherapy (docetaxel 60 mg/m^2 every 3 wk or irinotecan 150 mg/m^2 every 2 wk plus BSC) or BSC. The addition of second-line chemotherapy to BSC produced significant improvement in median OS (5.3 mo) when compared with BSC alone (3.8 mo) (HR = 0.657, 95%CI: 0.485-0.891; P = 0.007). The survival benefit remained consistent among the prospectively defined subgroups, including age, PS, number of prior treatments, metastatic sites, hemoglobin levels, and response to prior chemotherapy. No difference in median OS between docetaxel and irinotecan (5.2 mo *vs* 6.5 mo, P = 0.116) was registered. The most common toxicity of chemotherapy being myelosuppression and was easily manageable. Median relative dose-intensities for docetaxel and irinotecan arm were 95% and 93%, respectively. Moreover, patients in the chemotherapy arm more frequently received further treatment than those in the BSC arm (40% *vs* 22%, P = 0.011)[63].

In the Japanese WJOG 4007 phase Ⅲ trial[64], 223 patients with metastatic gastric cancer refractory to

Table 2 Phase Ⅱ studies on second-line therapy (combination therapy) in metastatic gastric cancer

Study	Total /eval pts	Performance Status, *n*	Ⅱ-line treatment, doses (mg/m^2)	Treatment ORR	Median TTP (mo)	Median PFS (mo)	Median OS (mo)	Toxicity, grade 3-4 (%)	Ref.
Irinotecan/ FU	40/38	ECOG 0/1/2 - 5/21/12	Iri/FA/FU - 180/125/400 bolus + 1200 inf over 22 h d1/d1/d1 q14	29.0%	NR	3.7	6.4	A/N/FN/I/N-E/D/As - 13/26/5/16/13/8/16	Assersohn *et al*[31], 2004
Irinotecan/ FU	64/57	ECOG 0/1/2 - 3/58/3	Iri/FU/FA - 150/100/1000 d1/d1/ d1, 2 over 24 h q14	21.0%	2.5	NR	7.6	N/T/D/E - 11/8/3/3	Kim *et al*[32], 2005
Irinotecan/ FU	51/48	ECOG 0-1/2 - 35/16	Iri/FA/FU - 180/200/400 bolus + 600 inf over 22 h d1/d1/d1 q14	18.0%	NR	3.2	9.1	A/N/FN/D/Nau/E - 14/17/1/6/6/4	[1]Seo *et al*[33], 2008
Irinotecan/ Cisplatin	32/31	ECOG 0/1/2 - 1/22/9	Iri/P - 70/30 d1, 15/ d1, 15 q28	15.6%	3.7	NR	6.1	A/N/T/E/D/M/Ano - 2.6/6/4.3/1.7/1.7/3.4/ 5.2	Baek *et al*[34], 2005
Irinotecan/ Cisplatin	87/70	ECOG 0/1/2 - 29/53/5	Iri/P - 70/80 d1, 15/ d1 q28	28.6%	4.3	NR	9.5	A/L/N/FN/ T/D/As/Nau - 28/34/40/10/8/6/5/2	[1]Takahari *et al*[35], 2010
Irinotecan/ Mitomycin C	38/38	KPS Median 80 (R 70-100%)	Iri/MMC - 150/8 d1, 15/d1 q28	32.0%	4.0	NR	8.0	A/L/N - 5/8/21	Giuliani *et al*[36], 2005
Irinotecan/ Mitomycin C	45	ECOG 0/1 - 24/21	Iri/MMC - 150/5 d1 q14	29.0%	NR	4.1	10.1	A/N/FN/D/I - 13/53/9/2/4	Hamaguchi *et al*[37], 2011
Irinotecan/ Capecitabine	48/46	ECOG 0/1/2 - 10/32/6	Iri/Cap - 100/1000 b.i.d d1, 8/d1-14 q21	27.1%	4.1	NR	7.6	N/FN/E/D/HFS - 8.7/4.3/4.3/17.4/4.3	Sun *et al*[38], 2009
Irinotecan/ Cetuximab	63/54	ECOG 0/1 - 28/35	Iri/Cet - 180/500 d1 q14	11.0%	NR	2.8	6.1	N/FN/D/F - 11/2/6/5	Schønnemann *et al*[39], 2012
Irinotecan *vs* Irinotecan/ mFOLFIRI	29 *vs* 30	ECOG 0/1 - 27/2 *vs* 27/3	Iri 150 d1 q14 Iri/mFOLFIRI 150 d1 + LV20 d1, 5-FU 2000 over 48 h	17.2% *vs* 20.0%	NR	2.2 *vs* 3	5.8 *vs* 6.7	A/n/Leu/FN/ Ano/As/D - 0/20/28/0/10/10/3 *vs* 10/13/37/3/13/3/7	Sym *et al*[40], 2013
Paclitaxel/ Doxifluridine	52/25	ECOG R 0-2	Dox/Ptx - 600 mg/70 d1-21/d7, 14, 21 q28	28.0%	NR	NR	5.8	L - 2	Arai *et al*[41], 2007
Paclitaxel/ Doxifluridine	33	ECOG 0/1/2 - 21/14/0	Dox/Ptx - 600 mg/70 d1-14/d1, 8 q21	18.2%	NR	4.0	10.7	A/L/N/D/ FN/I/As/Neu - 17.1/11.4/22.9/2.9/ 2.9/2.9/2.9	Takiuchi *et al*[42], 2008
Paclitaxel/ Carboplatin	50/47	ECOG 0-1/2 - 25/22	Ptx/Carbo - 175/AUC 5 d1/d1 q21	27.7%	NR	NR	OS in PR/SD - 10.0/6.0	N/T - 8.5/4.2	Stathopoulos *et al*[43], 2002
Paclitaxel/ Cisplatin	32/30	ECOG 0-1/2 - 21/11	Ptx/P - 145/60 d1 q21	25.0%	2.9	NR	9.1	N/E/Neu - 3/9/6	Lee *et al*[44], 2007
Paclitaxel/ Capecitabine	26/26	ECOG 0/1/2 - 7/11/8	Ptx/Cap - 175/825 b.i.d d1/d1-14 q21	34.6%	4.5	NR	7.5	A/N/T/HFS/Neu/Ar/ E - 3.8/11.5/3.8/11.5/ 11.5/7.5/3.8	Baize *et al*[45], 2009
Paclitaxel/ Capecitabine	36/35	KPS Median 80 (R 60-100%)	Ptx/Cap - 80/1000 b.i.d d1, d8/d1-14 q21	28.5%	NR	5.0	11.1	N/N-E/HFS - 11.1/5.6/5.6	Zhang *et al*[46], 2013
Paclitaxel/ S-1	30	ECOG 0-1/2 -23/7	Ptx/S-1 - 120 d1 q14 /80, 100, 120 mg/d, if BSA < 1.25 m^2, 1.26 m^2-1.49 m^2, ⩾ 1.50 m^2 b.i.d. d1-7 q14	33.3%	NR	3.6	7.2	A/Neu/T/Ano/Neu/ D/F - 73.4/63.4/16.6/20 /36.6/13.3/20	Zheng *et al*[47], 2014
Docetaxel/ Cisplatin	43/41	ECOG 0/1/2 - 1/33/9	Dtx/P - 60/60 d1/d1 q21	17.1%	NR	2.2	5.8	N/N-E/Neu/Neph - 29.3/12.2/4.9/2.3	Park *et al*[48], 2004
Docetaxel/ Cisplatin	30/30	KPS 50-70/ 80-100% - 14/16	Dtx/P - 60/60 d1/d1 q21	26.7%	4.5	NR	5.6	A/L/N/Nau - 3/27/27/3	Kunisaki *et al*[49], 2005
Docetaxel/ Cisplatin	32/32	ECOG 0/1/2 - 8/16/8	Dtx/P 70/70 d1/d1 q21	16.0%	5.0	NR	6.0	N/FN/T/D - 59/12/12/6	Polyzos *et al*[50], 2006
Docetaxel/ Oxaliplatin	38/37	ECOG 0/1/2 - 19/12/7	Dtx/Ox - 75/80 d1/d2 q21	10.5%	4.0	NR	8.1	N/As/Nau/Neu - 26.3/15.7/15.7/3	Barone *et al*[51], 2007
Docetaxel/ Oxaliplatin	48/46	ECOG 0/1/2 - 11/29/8	Dtx/Ox - 60/130 d1 q21	22.9%	4.4	NR	7.2	L/N/FN/T/ N-E/D/Neu - 17.4/26/7/4.3/28.3/ 15/6.5	Zhong *et al*[52], 2008
Docetaxel/ Epirubicin	50/45	ECOG 0/1/2 - 12/16/22	Dtx/Epi - 75/60 d1/ d1 q21	15.5%	2.4	NR	5.0	N/FN/T/N-E/ S/D/Neu - 68/48/46/2/8/4/2	Nguyen *et al*[53], 2006

Docetaxel/ Capecitabine	28/25	ECOG 0/1/2 - 2/19/7	Dtx/Cap - 60/1000 b.i.d d1/d1-14 q21	29.0%	4.0	NR	6.0	A/N/As/D/HFS - 7/36/7/11/7	Rosati *et al*[54], 2007
Docetaxel/ Etoposide	32/32	ECOG 0/1/2 - 6/20/6	Dtx/Eto - 75/50 d1/ d1-5 q21	9.4%	NR	3.0	6.0	N/FN/T/N-E/D/M - 29/19/3/15.6/9.4/6.2	Yildiz *et al*[55], 2010
Docetaxel/ Cetuximab	38/35	ECOG 0/1/2 - 15/21/2	Dtx/Cet - 30 d1, d15 q21/400 d1, then 250 d1 q7	6.0%	NR	2.1	5.4	I/FN/Ano/D/F - 11/3/16/11/29	Tebbutt *et al*[56], 2013
Mitomycin/ S1	43/33	ECOG 0/1/2 - 13/21/9	MMC/S-1 - 7/40 b.i.d d1/d1-28 q42	21.0%	NR	3.4	8.0	A/N/T/D/N-E/As/S - 7/5/5/10/7/12/10	Park *et al*[57], 2008
Mitomycin/ Capecitabine	39	ECOG 0/1/2/ UNK - 6/25/5/3	MMC/Cap - 10/1000 bid d1/d1-d14 q21	10.3%	NR	2.8	5.6	Leu/T/D/F/HFS/Neu - 5.4/10.8/5.4/8.1/5.4/8.1	Miranda *et al*[58], 2014
Methotrexate/ FU	56/55	ECOG 0/1/2/3 - 12/30/12/2	Mtx/FU/FA - 100 bolus/600 bolus (3 h after MTX)/10, every 6 h for 6 doses (24 h after MTX) q7	9.0%	NR	NR	7.8	A/L/N/T/N-E/D/S - 9.1/5.5/6.7/1.8/3.6/ 3.6/1.8	Hamaguchi *et al*[59], 2008
Cisplatin/ FU	58/53	ECOG 0-1/2 - 20/33	P/FU - 20/1000 over 20 h inf d1-5/d1-5 q28	11.3%	1.8	NR	4.6	A/N/T/N-E/D/M/ Neu - 8/9/9/11/4/6/1	Sencan *et al*[60], 2008
Oxaliplatin/ Sorafenib	40	ECOG 0/1/2 - 14/23/2	Ox/Sor - 130 d1 q21/400 mg b.i.d daily	2.8%	NR	3.0	6.5	N/T/As/D/Neu - 9.8/7.3/18/4.9/4.9	Martin-Richard *et al*[61], 2013

[1]Retrospective study. Cap: Capeceitabine; Carbo: Carboplatin; Cet: Cetuximab; Dox: Doxifluridine; Dtx: Docetaxel; Eto: Etoposide; Epi: Epirubicin; FU: 5-fluorouracil; FA: Leucovorin; Iri: Irinotecan; Mtx: Methotrexate; MMC: MitomycinC; Ox: Oxaliplatin; P: Cisplatin; Ptx: Paclitaxel; Sor: Sorafenib; ECOG: Eastern co-operative oncology group; KPS: Karnoffsky performance status; NR: Not reported; ORR: Objective response rate; OS: Overall-survival; Pts: Patients; PR: Partial response; PFS: Progression-free survival; R: Range; SD: Stable disease; TTP: Time-to-progression; UNK: Unknown; A: Anemia; As: Asthenia; Ano: Anorexia; D: Diarrhea; E: Emesis; FN: Febrile neutropenia; HFS: Hand-foot syndrome; I: Infection; L: Leucocytopenia; M: Mucositis; N: Neutropenia; Neu: Sensory neuropathy; Nau: Nausea; N-E: Nausea and vomiting; S: Stomatitis; T: Thrombocytopenia.

fluoropyrimidine and platinum combination treatment were randomly assigned to receive either paclitaxel (80 mg/m^2 on days 1, 8, and 15, every 4 wk) or irinotecan (150 mg/m^2 on days 1 and 15, every 4 wk). Nearly all patients had an ECOG PS of 0 or 1 (96%), the primary tumor was not resected in most patients (65%-66%), most had received prior S-1 plus cisplatin (79%-84%), and equal numbers of patients had intestinal or diffuse histology according to the Lauren classification. Patients with large volume ascites or bowel obstruction due to peritoneal carcinomatosis were excluded. After a median follow-up of 17.6 mo, OS was similar with paclitaxel and irinotecan [9.5 and 8.4 mo, (HR = 1.13, P = 0.38), respectively], as was progression-free survival (PFS) (3.6 and 2.3 mo [HR = 1.14, P = 0.33]) and response rate (21% and 14%, P = 0.24). Patients who received irinotecan experienced more grade 3-4 neutropenia (39.1% *vs* 28.7%) and diarrhea (4.5% *vs* 0.9%), whereas patients receiving paclitaxel experienced more grade 3-4 sensory neuropathy (7.4% *vs* 0%). The authors concluded that paclitaxel and irinotecan are reasonable second-line treatment options for metastatic gastric cancer[64].

In the COUGAR-02 phase 3 trial conducted in the United Kingdom[65], 168 patients with metastatic adenocarcinoma of the esophagus, gastro-esophageal junction, or stomach that had progressed on or within 6 mo of treatment with a platinum and fluoropyrimidine combination were recruited. Patients with an ECOG PS of 0 to 2 were randomly assigned to docetaxel 75 mg/m^2 every 3 wk for a maximum of 6 cycles plus BSC and BSC alone. After a median follow-up of 12 mo, median OS was modestly improved with docetaxel compared with BSC alone (5.2 mo *vs* 3.6 mo; HR = 0.67; P = 0.01). Responses were registered in 7% of assessable patients receiving docetaxel. Grade 3-4 toxicities in docetaxel included neutropenia (15%) leading to neutropenic fever (7%). Health-related quality-of-life measurements indicated no detrimental effect of chemotherapy and potential improvements in pain (P = 0.01) and nausea (P = 0.02)[65].

In an another Japanese phase Ⅲ study (TCOG GI-0801/BIRIP)[66], 130 patients with metastatic or recurrent gastric cancer refractory to S-1-based first-line chemotherapy were randomly assigned to receive BIRIP (irinotecan 60 mg/m^2 plus cisplatin 30 mg/m^2, every 2 wk) or irinotecan alone at a dose of 150 mg/m^2 every 2 wk. Less than 60% of patients had received platinum agents in the first-line setting. Enrolled patients had an ECOG PS score of either 0 or 1. With more than 70% of patients in Japan receiving third-line therapy, the primary endpoint of this study was the PFS benefit of the BIRIP regimen over irinotecan monotherapy. The median PFS was significantly longer in the BIRIP group than in the irinotecan group [3.8 mo *vs* 2.8 mo (HR = 0.68, P = 0.0398)]. The median OS was 10.7 mo in the BIRIP arm and 10.1 mo in the irinotecan arm (HR = 1.0, P = 0.9823). Although the response rates were not significantly higher in the BIRIP arm, when compared with irinotecan arm (22% *vs* 16%, P = 0.4975), the disease control rate was significantly better in the BIRIP group (75% *vs* 54%, P = 0.0162). The incidences of grade 3-4 safety events did not differ between the two groups. BIRIP had a good tolerability profile and was associated with no febrile neutropenia and less diarrhea. Thus, the BIRIP regimen significantly prolonged PFS compared with irinotecan alone. However, these results did not

Table 3 Phase Ⅲ studies on second-line therapy in metastatic gastric cancer

Ref.	Ⅱ-line treatment (mg/m^2)	Total pts	Performance Status, (ECOG/ WHO) (%)	Median ORR (%)	Median PFS (mo)	Median OS (mo)	Hazard ratio	Toxicity, grade 3-4 (%)
Thuss-Patience *et al*[62], 2011	Iri 250 cycle 1; 350 subsequent cycles	21/19	0-1/2 - 81/19	0	2.5	4.0	0.48, *P* = 0.012	D/FN/L/A - 26/16/21/11
	BSC	19	0-1/2 - 74/26	NR	NR	2.4		NR
Kang *et al*[63], 2012	Dtx 60 d1 q3w; Iri 150 d1 q2w	133	0/1 - 54/46	13.0	NR	5.3	0.657, *P* = 0.007	Doc: N/A/F/Ano/D/S - 15/30/26/6/3/3 Iri: N/A/F/Ano/D/S - 18/32/10/5/8/5
	BSC	69	0/1 - 52/48	NR	NR	3.8		N/A/F/D/S - 2/23/27/10/5/2
Hironaka *et al*[64], 2013	Iri 150 d1/d15 q4w	112	0-1/2 - 96.4/3.6	13.6[1]	2.3[1]	8.4	1.13, *P* = 0.38	L/N/A/FN/Ano/Neu/D/AST/Na - 19.1/39.1/30/9.1/17.3/0/4.5/8.2/15.5
	Ptx 80 d1/d8/d15 q4w	111	0-1/2 - 96.3/3.7	20.9[1]	3.6[1]	9.5		L/N/A/FN/Ano/Neu/D/AST/Na - 20.4/28.7/21.3/2.8/7.4/7.4/0.9/3.7/3.7
Ford *et al*[65], 2014	Doc 75 d1 q3w	84	0/1/2 - 28/55/17	7.0	29% (at 24 wk)	5.2	0.67, *P* = 0.01	N/A/FN/I/H/P/Neu - 15/6/7/15/1/11/4
	BSC	84	0/1/2 - 28/60/14	NR	NR	3.6		N/A/FN/I/H/P/Neu - 0/5/0/3/6/20/4
Higuchi *et al*[66], 2014	Iri 60 d1 q2w; Cis 30 d1 q2w	64	0/1 - 69/31	22.0[1]	3.8	10.7[1]	1.00, *P* = 0.982	N/A/FN/T/D/Ano/F - 13/16/0/0/2/6/3
	Iri 150 d1 q4w	66	0/1 - 68/32	16.0[1]	2.8	10.1[1]		N/A/FN/T/D/Ano/F - 36/18/5/2/6/11/6
Nishikawa *et al*[67], 2015	Iri 60 d1 q2w; P 30 d1 q2w	84	0/1 - 81/19	16.9[1]	4.6[1]	13.9[1]	0.834, *P* = 0.288	N/A/T/D/Ano/F - 35/16/1/0/6/9
	Iri 150 d1 q4w	84	0/1 - 75/25	15.4[1]	4.1[1]	12.7[1]		N/A/T/D/Ano/F - 28/4/0/3/9/4
Fuchs *et al*[69], 2014	Ram 8 mg/kg d1 q2w BSC	238	0/1/2 - 28/72/0	3.4[1]	2.1	5.2	0.776, *P* = 0.047	A/F/AbP/Dys/Dyn/Hyt/B/Prot/VTE - 6/6/6/2/2/8/3/< 1/1
	Placebo BSC	117	0/1/2 - 26/73/0	3.0[1]	1.3	3.8		A/F/AbP/Dys/Dyn/Hyt/B/Prot/VTE - 8/10/3/4/6/3/3//< 1
Wilke *et al*[70], 2014	Ram 8 mg/kg d1,15 q4w Ptx 80 d1/d8/d15 q4w	330	0/1 - 35/65	27	4.4	9.6	0.807, *P* = 0.017	N/A/Leu/T/F/Neu/D/H/Hyt/Prot/HepF/VTE - 41/9/18/2/8/4/4/15/1/4/2
	Placebo Ptx 80 d1/d8/d15 q4w	335	0/1 - 43/57	16	2.9	7.4		N/A/Leu/T/F/Neu/D/H/Hyt/Prot/HepF/VTE - 19/9/6/2/5/5/1/4/3/0/3/2
Satoh *et al*[76], 2014	Lap 1500 mg once daily Ptx 80 d1,d8,d15 q4w	132	0/1 - 45/55	27	5.5[1]	12.2[1]	0.84, *P* = 0.2	(experienced in > 10% of pts) N/A/Lym/F/Neu/D - 57/11/29/5/< 1/18
	Placebo Ptx 80 d1,d8,d15 q4w	129	0/1 - 37/63	9	4.4[1]	10.5[1]		(experienced in > 10% of pts) N/A/Lym/F/Neu/D - 31/6/2/< 1/0/2
Dutton *et al*[77], 2014	Gef 500 mg/d daily	224	0/1/2 - 25/52/22	24 (DC)	1.57	3.73[1]	0.90, *P* = 0.293	D/F/Sk/Rep/I/Hem/Vas/Met - 6/10/20/12/3/7/7/6
	Placebo	225	0/1/2 - 25/55/20	16 (DC)	1.17	3.67[1]		D/F/Sk/Rep/I/Hem/Vas/Met - 1/5/< 1/12/4/4/5/7

[1]The *P* value non-significant. BSC: Best Supportive Care; Dtx: Docetaxel; Gef: Gefitinib; Iri: Irinotecan; Lap: Lapatinib; P: Cisplatin; Ptx: Paclitaxel; Ram: Ramucirumab; DC: Disease control; ECOG: Eastern Co-operative Oncology Group; NR: Not reported; ORR: Objective Response Rate; OS: Overall-survival; PFS: Progression-free survival; Pts: Patients; WHO: World Health Organization; A: Anemia; AbP: Abdominal Pain; Ano: Anorexia; B: Bilirubin; D: Diarrhea; Dys: Dysphagia; Dyn: Dyspnea; E: Emesis; FN: Febrile neutropenia; H: Hemorrhage; Hem: Hematologic toxicity; HepF: Hepatic failure; Hyt: Hypertension; I: Infection; L: Leucocytopenia; Ly: Lymphopenia; M: Mucositis; Met: Toxicity associated with metabolism and nutrition; N: Neutropenia; Neu: Sensory neuropathy; Nau: Nausea; N-E: Nausea and vomiting; P: Pain; Prot: Proteinuria; Resp: Respiratory toxicity; Sk: Skin toxicity; T: Thrombocytopenia; Vas: Vascular toxicity; VTE: Venous thromboembolic events.

translate to the overall survival benefit[66].

A recently published Japanese phase Ⅲ randomized study (TRICS)[67] included platinum-naïve patients progressing following S-1 monotherapy for metastatic gastric cancer or relapsed within 6 mo after completion of S-1 adjuvant therapy. Approximately 168 patients with an ECOG PS of 0 or 1 were randomly allocated to irinotecan 60 mg/m^2 and cisplatin 30 mg/m^2 every 2 wk (n = 84) or irinotecan 150 mg/m^2 every 2 wk (n = 84). No significant differences were observed in the OS (13.9 *vs* 12.7 mo, HR = 0.834, 95%CI: 0.596-1.167), PFS (4.6 *vs* 4.1 mo, HR = 0.860, 95%CI: 0.615-1.203) and response rates (16.9% *vs* 15.4%, P = 0.812). The favorable long-term survival rates observed in

this study may be due to the favorable prognostic characteristics of the patients, as only patients with an ECOG PS of 0-1 were considered, the median number of metastatic sites was 1, and only 21% of patients had 2 or more metastatic sites. Compared to the irinotecan arm, the irinotecan/cisplatin arm showed significantly higher grade 3-4 toxicities, regarding anemia (16% *vs* 4%) and lactate dehydrogenase level (5% *vs* 0%). There was no survival benefit observed upon adding cisplatin to irinotecan after failure of S-1 monotherapy[67].

The meta-analysis by Kim *et al*[68] demonstrated evidence to support the efficacy of second-line chemotherapy in the treatment of metastatic gastric cancer. The meta-analysis pooled together 410 patients from three randomized trials[62,63,65] evaluating chemotherapy (docetaxel/irinotecan) versus BSC as second-line therapy in patients with metastatic gastric cancer. This meta-analysis demonstrated that there exists a clear survival benefit with a 36% reduction in the risk of death when using second-line chemotherapy in patients with metastatic gastric cancer (HR = 0.64; 95%CI: 0.52-0.79, $P < 0.0001$). The observed results were consistent irrespective of the administered drugs, and the extent of survival benefit was similar between European and Asian populations (HR = 0.63 and 0.657, respectively)[68].

Biological agents: With the arrival of ToGA study data, a new era of targeted therapy was opened for gastric cancer[18]. Ramucirumab is the first biological agent that showed a survival benefit in patients with metastatic gastric or gastro-esophageal junction adenocarcinoma progressing after first-line chemotherapy, given either as a single drug[69] or in combination with paclitaxel[70]. Ramucirumab is a fully human Immunoglobulin G1 monoclonal antibody receptor antagonist designed to bind the extracellular domain of Vascular Endothelial Growth Factor (VEGF) Receptor-2, thereby blocking the binding of VEGF ligands and inhibiting receptor activation and thus inhibiting angiogenesis[71].

In the phase Ⅲ randomized study (REGARD)[69], 355 patients from all parts of the world who progressed after platinum- or fluoropyrimidine-based first-line chemotherapy were randomized to receive either ramucirumab 8 mg/kg on day 1 every 2 wk (n = 238) or placebo (n = 117). Almost all the patients had an ECOG PS of 0-1. In the active treatment arm, 65% of patients had a progression-free interval of ≥ 6 mo from the end of the previous treatment. Single agent ramucirumab showed a significant survival benefit when compared with the placebo arm (median OS - 5.2 *vs* 3.8 mo, HR = 0.776, $P = 0.047$). Estimated rates of 12-wk PFS were 40.1% in patients in the ramucirumab arm and 15.8% in the placebo arm. The rate of disease control was significantly higher in patients given ramucirumab than in those given placebo (49% *vs* 23%). The median time to deterioration in ECOG PS to a score of 2 or worse was 5.1 mo in the ramucirumab arm and 2.4 mo in the placebo arm (HR = 0.586, $P = 0.002$). With regard to safety, more patients in the ramucirumab group had grade ≥ 3 hypertension than those in the placebo group (8% *vs* 3%)[69].

In another large phase Ⅲ randomized study (RAINBOW)[70], 655 patients with a PS score of 0-1 received either ramucirumab 8 mg/kg (n = 330) or placebo (n = 335) on days 1 and 15, plus paclitaxel 80 mg/m² on days 1, 8, and 15 of a 28-d cycle. In both arms, 75% of patients had a time-to-progression duration of < 6 mo on first-line therapy. More than 70% and 20% of patients received a doublet and triplet regimen in first-line therapy (containing both fluoropyrimidines and platinum agents), respectively. Median OS (9.6 mo *vs* 7.4 mo. HR = 0.807, $P = 0.017$) and median PFS (4.4 mo *vs* 2.9 mo. HR = 0.635, $P \leq 0.001$) were significantly increased in the ramucirumab plus paclitaxel arm compared with the placebo and paclitaxel arm. Preplanned forest plot analyses showed an OS benefit in all subgroups. Grade ≥ 3 adverse events that occurred more often in the Ramucirumab plus Paclitaxel arm included neutropenia (41% *vs* 19%), hypertension (14% *vs* 2%), fatigue (12% *vs* 5%), anemia (30% *vs* 10%), and abdominal pain (6% *vs* 3%). Thus, the addition of ramucirumab to paclitaxel significantly increased OS, and this regimen could be considered as a new standard of second-line treatment for patients with metastatic gastric cancer[70].

Antiangiogenic therapy is not solely limited to monoclonal antibodies. In addition to ramucirumab, there are several other agents, such as apatinib and regorafenib, that exhibit an antiangiogenic effect. Apatinib is an oral VEGFR2 tyrosine kinase inhibitor (TKI). The results of a randomized phase Ⅲ study conducted in China that investigated the survival benefit of apatinib over placebo were presented in 2014. Patients who failed to improve after two prior lines of treatment were randomly assigned to receive apatinib (n = 180, 850 mg once daily) and placebo (n = 90). The median OS was significantly higher in the apatinib arm compared to the placebo arm (195 *vs* 140 d, HR = 0.71, $P < 0.016$) as well as PFS (78 *vs* 53 d, HR = 0.44, $P < 0.0001$)[72].

In 2015, the preliminary results of a randomized phase Ⅱ study (INTEGRATE), which studied the efficacy and safety of regorafenib over BSC in metastatic gastric cancer patients who failed to improve after one or two lines of chemotherapy, were reported. Regorafenib is an oral small-molecule inhibitor of multiple protein kinases, including those involved in angiogenesis (VEGFR 1, 2, and 3, TK with Ig and EGF homology domain 2), oncogenesis (KIT, RET, RAF-1, BRAF), and the tumor microenvironment (platelet-derived growth factor receptor-β, fibroblast growth factor receptor)[73]. A total of 152 patients were randomly assigned to receive regorafenib (160 mg, days 1-21 every 28 d) plus BSC over placebo plus BSC

in a 2:1 fashion. The median PFS was improved in the regorafenib arm compared with placebo (11.1 wk *vs* 3.9 wk, HR = 0.41 $P < 0.0001$). OS data have not yet been reported. Regorafenib therapy was well tolerated, and there were no new safety signals[74].

Lapatinib, a EGFR1-2 TKI, did not show survival benefit in first-line therapy when combined with capecitabine plus oxaliplatin in HER2-positive advanced or metastatic gastric and esophageal adenocarcinomas in the TRIO-013/LOGiC trial[75]. Lapatinib was also studied in a phase Ⅲ randomized (TyTAN) study in second-line treatment for an Asian population[76]. Patients deemed HER2-positive by FISH (n = 420) were either randomized to receive lapatinib (1500 mg once daily) plus paclitaxel (80 mg/m^2 on days 1, 8, 15 every 4 wk) or paclitaxel alone. In the intent-to-treat (ITT) population, the median OS was improved in the experimental arm from 8.9 to 11 mo (HR = 0.84; P = 0.2088). There was no significant difference in the median PFS (5.4 mo *vs* 4.4 mo) or TTP (5.5 mo *vs* 4.4 mo). Around one-third of patients had tumors with no HER2 expression (0/1+) according to immunohistochemistry (IHC) in both arms. Pre-planned subgroup analysis showed a significant OS benefit in patients with IHC 3+ expression treated with lapatinib (14 mo) compared to those treated with paclitaxel alone (7.6 mo, HR = 0.59; P = 0.0176)[76].

Gefitinib, an EGFR1 TKI, was investigated in the second-line treatment of metastatic esophageal cancer or type Ⅰ/Ⅱ Siewert junctional tumors in a phase Ⅲ randomized trial (COG study). Approximately 450 patients with a squamous-cell carcinoma or adenocarcinoma with WHO PS 0-2 were randomly assigned to receive gefitinib 500 mg/d or placebo. Gefitinib resulted in a statistically significant improvement of PFS (HR = 0.8), but not OS (3.73 mo *vs* 3.67 mo, HR = 0.90, P = 0.29)[77].

Everolimus is an oral mammalian target of rapamycin (mTOR) inhibitor. Everolimus was studied in a phase Ⅲ randomized study (GRANITE-I) in metastatic gastric cancer patients progressing after one or two lines of previous systemic chemotherapy. A total of 656 patients were randomized in a 2:1 fashion to everolimus plus BSC (n = 439) or placebo plus BSC (n = 217). In both arms, 48% of patients had received one previous therapy and 52% had received two previous therapies. Compared with BSC, everolimus did not significantly improve survival (median OS - 5.4 mo *vs* 4.3 mo; HR = 0.90, P = 0.124)[78].

FUTURE DIRECTIONS

In the second-line treatment of metastatic gastric cancer, factors such as hepatocyte growth factor receptor (c-Met), fibroblast growth factor receptor (FGFR), epithelial cell adhesion molecule (EpCAM), insulin-like growth factor receptor 1 (IGF-1R), phosphatidylinositol 3-kinases (PI3K), cyclin dependent kinases (CDK), mitogen-activated protein kinases (MAPK), immune checkpoints (PD-1 and PD-L1), matrix metalloproteinases, proteasomes, histone deacetylases, chaperone proteins, and other molecular structures are under evaluation. Novel drugs directed against those specific targets are under clinical investigation.

Preclinical data suggest that the hepatocyte growth factor (HGF)/MET pathway may represent a therapeutic target for gastric adenocarcinoma[79,80]. The expression of receptors to HGF is found in up to 74% of cases of gastric adenocarcinoma. However, mutations in the c-Met gene are found in 10% of cases, and gene amplification is found in 2%-23% of gastric tumors[81-85]. C-Met overexpression has been associated with poor prognosis. Signals sent from the HGF receptor activate a wide range of cellular signaling pathways, which promote proliferation, migration and survival. This has made c-Met a potential therapeutic target. The c-Met inhibitors crizotinib and foretinib did not show significant activity in c-Met gene-amplified gastric cancer[84,86]. However, monoclonal antibodies to c-Met, including rilotumumab and onartuzumab, are being actively studied in phase Ⅲ studies[87,88] in the first-line treatment of metastatic gastric cancer. Rilotumumab showed promising results in a randomized phase Ⅱ placebo-controlled study. Approximately 121 patients with metastatic gastric cancer receiving first-line therapy with epirubicin, cisplatin and capecitabine (ECX) were randomly assigned to receive rilotumumab or placebo (40 to rilotumumab 15 mg/kg; 42 to rilotumumab 7.5 mg/kg; 39 to placebo). ECX plus rilotumumab significantly reduced the risk of disease progression compared with placebo (HR = 0.6, P = 0.016). However, there was no significant difference in overall survival. It should be noted that only 56% of the patients included in the study had a tumor expressing the c-Met gene[89]. The results of two other studies on c-Met inhibitors were presented in ASCO GI 2015[90,91]. AMG337, a selective inhibitor of the tyrosine kinase c-Met, was investigated in a phase Ⅰ study in patients with gene amplification. An objective response was achieved in 8 out of 13 (62%) patients with gastric cancer[90]. Onartuzumab, a monoclonal antibody to c-MET, was studied in combination with a FOLFOX regimen in a randomized phase Ⅱ study, which included 123 patients with metastatic gastric cancer. The study did not improve the long-term results. The most possible explanation for the study's failure could be the patient selection, which was based on overexpression rather than gene amplification[91]. Thus, the blockade of c-met has become an extremely promising strategy in the targeted therapy of gastric cancer.

In recent years, agents targeting immune checkpoints (PD-1 and PD-L1) are gaining momentum in oncology. Programmed cell death-1 (PD-1), an immunoinhibitory receptor of the CD28 family, plays a major role in the immune escape of tumors[92]. One of the mechanisms by which tumors evade host T

cells is by activating immune checkpoints that block T-cell activation. The presence of PD-L1 on tumor cells allows them to escape the cytotoxic effects of immune cells. Pembrolizumab, a highly selective humanized IgG4/kappa isotype monoclonal antibody that blocks PD-1's interaction with its ligands PD-L1 and PD-L2, was approved for the treatment of metastatic melanoma in 2014. Pembrolizumab was studied in a phase Ib study, which was presented at the ESMO meeting in 2014[93]. Among 39 patients with chemotherapy-refractory metastatic gastric or gastro-esophageal junction carcinoma and PD-L1 expression in $\geqslant$ 1% of tumor cells, pembrolizumab administration achieved an objective response of 31% with a median response duration of 6 mo. There was a correlation between the degree of expression of PD-L1 and objective tumor response[93]. Following these encouraging results, a randomized phase Ⅱ study has been initiated to investigate the efficacy of pembrolizumab in monotherapy or in combination with cisplatin and 5-fluorouracil for the first-line treatment of metastatic gastric cancer.

BRCA mutations in gastric cancer are extremely rare. However, the decrease in the activity of certain components of homologous recombination occurs in 35%[94]. Changes in the activity of other molecules are not directly associated with the process of homologous recombination, up to 70% (PTEN dysfunction, mutation of p53 and ERCC1)[95-97]. Preclinical data indicate that olaparib, an oral poly (ADP-ribose) polymerase inhibitor, showed increased efficiency when combined with chemotherapeutic agents[98]. A randomized phase Ⅱ study compared the efficacy of olaparib (100 bid, daily) (n = 61) or placebo (n = 62) in combination with paclitaxel (80 mg/m^2 days 1, 8, 15 per 28-day cycle) on both arms as second-line therapy in patients with recurrent/metastatic gastric cancer. Investigators found that in the absence of ATM expression in tumors, which constitute the surrogate of homologous recombination deficiency, the addition of olaparib to paclitaxel significantly increased OS, but not PFS[99]. This has created a new dimension of research in the targeted therapy of gastric cancer - the influence on the DNA repair mechanisms in tumors.

Currently, one of the most studied biomarkers in oncology is the therapeutic targets for fibroblast growth factor receptors (FGFR) and their ligands. The family of human FGF comprises 22 proteins and 5 types of receptors for FGF (FGFR). The FGF/FGFR complex is involved in the differentiation and proliferation of various cells[100,101]. In gastric cancer, the gene amplification of FGFR4 is associated with poor prognosis[102,103]. As in other tumor types, the presence of the FGFR4 gene polymorphism Gly388Arg has proved to be of prognostic significance in gastric cancer[104]. In a Japanese study[105], which included tumor samples from 222 gastric cancer patients, high levels of expression of FGFR 1, 2, 3, and 4 (without amplification) were detected in 30%, 51%, 64% and 79% of tumors, respectively. The overexpression of FGFR1, FGFR2 or FGFR4 was found to be significantly associated with tumor progression, including depth of invasion, lymph node metastasis, pathological stage and distant metastasis or recurrent disease. Therefore, FGFR-targeted therapeutics using small-molecule compounds that inhibit the binding of FGF to FGFR is a promising direction for research. For example, the inhibition of FGFR2 signaling by AZD4547, a FGFR inhibitor, resulted in significant dose-dependent tumor growth inhibition in FGFR2-amplified gastric cancer cell lines[106]. AZD4547 is currently being studied in a randomized phase Ⅱ trial as monotherapy and in combination with paclitaxel for the second-line treatment of patients with metastatic gastric or gastro-esophageal junction cancer with FGFR2 polysomy or gene amplification[107].

DISCUSSION

Although data from randomized clinical trials show an increased survival benefit with second-line therapy, not all patients are offered second-line therapy in real-life clinical practice. This could possibly be explained by the poor PS, which the majority of patients experience with disease progression after first-line therapy. In Japan, almost all patients with metastatic gastric cancer receive second-line therapy and more than 50% of patients receive three lines of therapy. However, in western countries, only half of the patient population is offered second-line treatment on progression after first-line therapy. These regional ethnic differences should be taken in to consideration before translating the survival benefit from clinical studies to real-life practice. Additionally, the OS benefit obtained from phase Ⅲ studies was observed in selected patients who had adequate organ function and no severe co-morbidities at the time of entry into the study.

In order to obtain a clear survival benefit, it would be more rational to use second-line therapy in a separate group of patients with higher predictive survival rate who are likely to benefit from second-line therapy. This would allow us to spare the adverse effects of cytotoxic agents in patients with lower predictive survival rates. Of the randomized clinical trials[62,63,69], several factors, such as poor performance status, presence of peritoneal metastases, gastro-esophageal location of the primary tumor and a chemotherapy-free interval of < 3 mo were identified as clinicopathological prognostic factors for reduced OS. Moreover, several other retrospective studies identified similar prognostic factors (performance status, time to progression of first-line chemotherapy and hemoglobin levels)[25,33,108-110]. Therefore, factors such as the patient's general condition, metastatic extent, previously used cytotoxic agents, toxicity profile, cumulative toxicity, lack of cross-resistance to previously used agents, and previous response to first-line therapy should always be considered while

administering second-line therapy. The role of second-line therapy in metastatic gastric cancer should not only be improving OS but also achieving better symptom control and improved QoL. Park *et al*[111] found that second-line therapy by itself improves the QoL and Hospital Anxiety and Depression Scale (HADS) scores in patients with metastatic gastric cancer, regardless of the objective tumor response. This shows that second-line therapy is justified in patients who are physically fit and willing to receive further chemotherapy. These findings are different from the traditional endpoints such as tumor response rate and survival rate.

A recent meta-analysis[112] analyzed the published phase Ⅲ trials[62,63,65,69,78] that compared active treatment to BSC in metastatic gastric cancer. Both chemotherapy and ramucirumab had similar activity in terms of the reduction of the risk of death by 27% and 22%, respectively. This analysis demonstrated that a significant OS benefit was registered with active second-line treatments (irinotecan, docetaxel, ramucirumab), even in patients with impaired performance status [ECOG $\geqslant$ 1, HR = 0.82 (0.79-0.85)][112]. This suggests that patients with symptomatic disease should not be excluded from further lines of treatment following the failure of first-line therapy. It should be noted that for the first time in the second-line setting, the addition of a targeted agent (ramucirumab) to standard chemotherapy (paclitaxel) demonstrated a significant survival benefit by increasing the median OS from 7.4 to 9.6 mo (P = 0.017) and median PFS from 2.9 to 4.4 mo. However, the other biological agents, such as everolimus, lapatinib and gefitinib, failed to show a survival benefit when administered as second-line therapy. In addition to HER2 overexpression, to date, there are no predictive biomarkers in the treatment of metastatic gastric cancer. This has reemphasized the fact that clinical studies with better patient selection based on predictive biomarkers are necessary.

Older microarray studies[113-117] performed in gastric cancer cell lines described the expression changes associated with morphological and tissue type differences in gastric cancer. This approach has changed as pathway signatures (rather than individual genes) are used as the basis for cancer classification. Recently, the Cancer Genome Atlas (TCGA) project[118] proposed a molecular classification dividing gastric cancer into four genomic subtypes: Epstein-Barr virus-infected tumors, which display recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, PD-L1/2; microsatellite unstable tumors, which show elevated mutation rates, including mutations of genes encoding targetable oncogenic signaling proteins; genomically stable tumors, which are enriched for the diffuse histological variant and mutations of RHOA or fusions involving RHO-family GTPase-activating proteins; and chromosomally unstable tumors, which show marked aneuploidy and focal amplification of receptor tyrosine kinases. This classification has provided a roadmap for better patient stratification for clinical studies that are to be planned with targeted agents.

CONCLUSION

In the second-line setting, to date, three agents (irinotecan, taxane and ramucirumab) have shown a survival benefit over BSC or active agents in randomized phase Ⅲ studies. Furthermore, a paradigm shift from disease-specific new drug development to biomarker-oriented investigations is gaining momentum in the treatment of metastatic gastric cancer. Clinical trials of molecular targeted agents should focus on specific patient subsets. This will result in individualizing therapeutic strategies by maximizing drug efficacy and minimizing adverse effects in patients with metastatic gastric cancer.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Emerging blood-based biomarkers for detection of gastric cancer

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Abstract

Early detection and efficient monitoring of tumor dynamics are prerequisites for reducing disease burden and mortality, and for improving the management of patients with gastric cancer (GC). Blood-based biomarker assays for the detection of early-stage GC could be of great relevance both for population-wide or risk group-based screening programs, while circulating biomarkers that reflect the genetic make-up and dynamics of the tumor would allow monitoring of treatment efficacy, predict recurrences and assess the genetic heterogeneity of the tumor. Recent research to identify blood-based biomarkers of GC has resulted in the identification of a wide variety of cancer-associated molecules, including various proteins, autoantibodies against tumor associated antigens, cell-free DNA fragments, mRNAs and various non-coding RNAs, circulating tumor cells and cancer-derived extracellular vesicles. Each type of these biomarkers provides different information on the disease status, has different advantages and disadvantages, and distinct clinical usefulness. In the current review, we summarize the recent developments in blood-based GC biomarker discovery, discuss the origin of various types of biomarkers and their clinical usefulness and the technological challenges in the development of biomarker assays for clinical use.

Key words: Gastric cancer; Biomarker; Liquid biopsy; Cell-free DNA; Cell-free RNA; Extracellular vesicles; Autoantibodies; Proteomics

Core tip: The identification of blood-based biomarkers that could reliably detect the presence of early-stage gastric cancer or provide means to monitor the tumor dynamics is an unmet clinical need. Recently, considerable effort has been devoted to discovering various types of cancer-associated molecules in the blood of gastric cancer patients, and this has resulted in establishing biomarker models with remarkably high sensitivity and specificity. However, a validation in large-scale studies and a head-to-head comparison of the biomarker models and technologies are required before these biomarkers can be used in routine clinical practice.

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INTRODUCTION

Although the incidence of gastric cancer (GC) has decreased in most parts of the world, with estimated 952000 new cases and 723000 deaths from GC in 2012, it still accounts for approximately 6.8% of all cases and 8.8% of cancer-related deaths worldwide[1]. The incidence rates vary significantly across the globe, being the highest in Eastern Asia, followed by Central and Eastern Europe and rates are the lowest in North America and Western Africa[1,2]. The main type of GC is adenocarcinoma (approximately 95%), which can be further categorized into an intestinal and a diffuse type according to Lauren's classification[3]. Intestinal-type gastric adenocarcinoma, the most common subtype of GC, develops through a well-described sequence of histopathological stages from normal mucosa to chronic gastritis, chronic atrophic gastritis followed by intestinal metaplasia, dysplasia and finally to adenocarcinoma, with *H. pylori* infection, which is recognized as the main underlying cause of pan-gastric mucosal inflammation[4,5]. Thus, the main risk factors for GC are chronic infection with *H. pylori* and the presence of the above-listed precancerous lesions, whereas a relatively smaller proportion of GC cases are linked to a genetic predisposition and dietary factors[6,7].

The high mortality rate in GC mostly results from its detection at late stages. Most GC cases are detected at stage ⅢA-Ⅳ, when the estimated 5-year survival ranges from 7%-27% and the median survival is less than 12 mo[8,9]. On the contrary, early GC that is limited to the submucosal layer is curable by endoscopic mucosal dissection or minimally-invasive surgery[10]. Early GC detection, however, is hampered by the lack of specific symptoms before it has spread beyond the original site. Thus, organized screening programs that aim to detect pre-cancerous lesions and early-stage GC seem to be a main tool for reducing GC-related mortality, but such programs have been implemented only in some Asian countries[11]. Upper endoscopy is the primary screening technique in most of the programs and the gold standard for confirmation of the diagnosis[6,11]. However, endoscopy is an invasive technique with uncommon but serious side effects and a relatively high cost, and the results are highly dependent on the skill of the endoscopist[6]. Therefore, GC screening in low GC incidence areas and low-income countries is not practical, and is likely to be associated with low participation rates in the screening programs.

Currently, the only non-invasive test that has been used for GC detection is the pepsinogen (PG) test. PGs are pro-enzymes that are converted into the proteolytic enzyme pepsin. PGs are mainly synthesized and secreted by the gastric chief cells and their serum levels indirectly reflect secretion in the stomach[12]. PGI is exclusively produced by the corpus mucosa, while PGⅡ is also secreted by the cardiac and pyloric glands and the proximal duodenal mucosa[13]. Low PGI levels and a low PGⅠ/PGⅡ ratio are indicators of atrophic changes in the gastric corpus. PG tests can detect gastric mucosal atrophy with a sensitivity of 66.7%-84.6% and a specificity of 73.5%-87.1%[14-16], whereas the sensitivity for GC detection ranges from 36.8%-62.3%[17-19], which is not acceptable in population-based screening settings. Thus, the PG test can be administered in a two-stage screening approach as a primary screening test to identify individuals who are at an elevated GC risk, and these high-risk individuals are then referred for endoscopic examination followed by the histological analysis of gastric biopsy[11].

In recent years, considerable effort has been devoted to the discovery of novel blood-based biomarkers that are suitable for the development of non-invasive tests to detect GC at an early stage or to monitor tumor dynamics. Such biomarkers may include quantitatively- or structurally-altered proteins, cancer-associated autoantibodies, cell-free nucleic acids (cfNAs), circulating tumor cells (CTCs), cancer-derived extracellular vesicles (EVs) and metabolites. In the current review, we provide an overview of recently-discovered blood-based GC biomarkers, and discuss their origin and mechanisms of release into the bloodstream, and also their potential clinical usefulness.

CRITERIA FOR BIOMARKERS APPLICABLE TO CANCER CONTROL PROGRAMS

In 2013, a Working Group of international experts established by the International Agency for Research on Cancer made recommendations for GC control

and concluded that a decisive public health action to include GC in cancer control programs is required; however, interventions should be tailored to the local conditions, taking into account the prevalence, cost-benefit ratio and adverse consequences[20]. Prevention strategies should aim to reduce both GC incidence and mortality. Primary prevention strategies are focused on preventing exposure to GC risk factors, for example, by eradicating *Helicobacter pylori* (*H. pylori*) infection or modifying patients' diet and lifestyle, while secondary prevention strategies aim to identify patients with early-stage GC or precancerous lesions, who would then undergo endoscopic surveillance[6,11]. Tertiary prevention aims to control the symptoms and morbidity of established cancer. Blood-based biomarkers for the detection of early-stage, residual or recurrent cancers could be highly relevant for both secondary and tertiary prevention strategies.

Ideally, a biomarker that is used in population-wide screening programs should be stable and robustly measurable in plasma or serum using routine laboratory equipment, appear in the bloodstream before the clinical signs and symptoms arise, should discriminate between cancer and inflammatory diseases and should have high positive and negative predictive values. However, the relatively low prevalence of GC in most parts of the world, except for Eastern Asia, suggests that even biomarker assays with high sensitivity and specificity would have a low positive predictive value (PPV). For example, if a hypothetical biomarker assay with a sensitivity of 95% and specificity of 98% would be applied to screen 100000 asymptomatic individuals in a medium-incidence area such as Eastern Europe (with GC prevalence of 0.04%), 38 true positives, 2 false negatives and approximately 2000 false positives would be detected, thus yielding PPV of only 1.87%.

A biomarker for detecting residual or recurrent cancer, however, must reflect the tumor dynamics. For example, it should be rapidly cleared from the circulation after complete tumor removal, and it should be able to detect incompletely-resected tumor and to increase in the circulation before the clinical signs of recurrence.

PROTEOMIC BIOMARKERS

Proteomic analyses can provide information on a complex composition of proteins that are differentially expressed in blood specimens from cancer patients and healthy donors that could be used for cancer biomarker discovery. The flowchart of serum proteomic analysis usually consists of protein extraction and separation performed by 2-dimensional gel electrophoresis (2-DE), difference gel electrophoresis (2D-DIGE), surface-enhanced laser desorption/ionization (SELDI), Liquid Chip and other approaches. These are followed by diagnostic model determination or protein identification through MS and bioinformatics, after which identified proteins are verified using conventional techniques such as Western blot and ELISA (technology approaches reviewed by Liu *et al*[21]). The current challenges in blood-borne biomarker discovery include variability of sample preparation and pre-treatment as well as inter-laboratory analytical variability of different instruments used in discovery and validation studies. Another issue is the choice of sample type used for proteome analyses - serum seems to be the most common choice because of its availability in biobanks and thus, it is frequently used in studies. However, the Human Proteome Organisation recommends the use of plasma for proteomic studies to reduce the variability caused by the coagulation process[22].

Many proteomic studies of serum biomarkers for GC detection have been published in the last 10 years (reviewed in detail by Liu *et al*[21] and Lin *et al*[23]), and examples of biomarker models are listed in Table 1. In one of the pioneering studies, Ebert *et al*[24] analyzed serum from GC patients with SELDI-TOF-MS and Protein-Chip technology in combination with a pattern-matching algorithm and built a classifier ensemble that consists of 50 decision trees that achieved 100% sensitivity and 96.7% specificity (including both, intestinal and diffuse type GC). Moreover, this classifier could detect early stage GC with sensitivity of 89.9%. Liu *et al*[25] showed that there were three differentially-expressed peaks identified by screening serum samples from 65 GC and 53 cancer-free individuals, including patients with chronic superficial gastritis and chronic atrophic gastritis. The combined use of the three biomarkers, which were identified as fibrinogen α chain, apolipoprotein A-Ⅱ and apolipoprotein C-I, distinguished the cancer group from the control group with a sensitivity of 93.85% and a specificity of 94.34% in an independent validation set. In another study, Li *et al*[26] found a six-feature proteomic model by applying SELDI-TOF-MS analysis that effectively distinguished GC samples from control samples with a sensitivity of 93.5% and specificity of 91.6%. In addition, they observed that three of the peaks were differentially expressed between patients with stage Ⅰ GC and advanced GC (accuracy 88.9%). Other groups have reported using the SELDI-MS application to analyze the serum profile from GC patients, and they showed an overall high sensitivity and specificity (over 90% and 80%, respectively)[27-32]. However, these promising results have to be validated in larger multicenter studies because the SELDI-MS approach has several disadvantages, as follows: the results lack consistency among research groups, the reproducibility is low and it cannot directly identify proteins[33].

Other approaches besides SELDI-MS have been used. Yang *et al*[34], using magnetic beads, separated peptidome from GC patients' serum using matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) MS, and they found 11 differentially-expressed proteins and the two most promising of them could detect GC patients with 95.2% sensitivity

Table 1 Proteomics-based biomarker models for detection of gastric cancer

Biomarker model	Approach used	Sample size and type (cancer/controls)	Diagnostic value[1]	Ref.
Five peaks - 3316, 6629, 3217, 3952, 6431 Da	MB-WCX, MALDI-TOF-MS	T: GC = 32/HC = 32 V: GC = 30 (GC Ⅰ-Ⅱ = 8)/HC = 30	AUC = 0.86-0.99 for individual features (P < 0.001), Sn = 79.3%, Sp = 86.5% Sn = 71.7% for early stage GC	Yang *et al*[148], 2012
1546 Da (SERPINA1) 5335 Da (ENOSF1)	MB-WCX, MALDI-TOF-MS; ELISA for validation	T: GC = 70/HC = 72 V: GC = 36/HC = 36, BGD = 30, other cancers = 108	AUC (1546 Da) = 0.83 (P < 0.001), AUC (5335 Da) = 0.87 (P < 0.001) - calculated for training set; in validation set SERPINA1 concentration was significantly higher for GC patients than for all other controls (P < 0.001) and ENOSF1 concentration was significantly higher for GC patients than HC (P < 0.001)	Yang *et al*[34], 2015
Fibrinogen α-chain, apolipoprotein A-Ⅱ and apolipoprotein C-Ⅰ	HPLC, LC-MS/MS	T: GC = 65/HC = 30, BGD = 23 V: GC = 44/ HC = 30, BGD = 23	Sn = 90.9%, Sp = 90.6% (P = NA)	Liu W *et al*[37], 2012
Six peaks at 2873, 3163, 4526, 5762, 6121 and 7778 m/z; For stage Ⅰ three peaks at 2873, 6121 and 7778 m/z	Protein Chip SELDI-TOF-MS	GC = 169 (GC Ⅰ = 27)/ HC = 83	Sn = 93.5%, Sp = 91.6% Accuracy for stage Ⅰ - 88.9%, (P = NA)	Li *et al*[26], 2012
EGFR, proApoA1, ApoA1, TTR, RANTES, VN, DD, IL-6, A2M, CRP, PAI1	xMAP (Luminex), ELISA	T: GC = 120/BGD = 101, HC = 19 V: GC = 95 (GC Ⅰ-Ⅱ = 75)/BGD = 43, HC = 8	AUC = 0.95, (P < 0.05) Sn = 88.8%, Sp = 89.7% Sn (Ⅰ-Ⅱ) = 92.3% Sn (tumor size ⩽ 2 cm) = 81.8%	Ahn *et al*[35], 2012
Four peaks at 1867 (tubulin beta chain), 2701 (thymosin beta4 like protein3), 2094 (cytochrom b-c1 subunit), 1467 Da	MB-WCX, MALDI-TOF-MS	T: GC = 40/HC = 39 V: GC = 40/GA = 30, HC = 39	AUC (1867 Da) = 1, AUC (1467 Da) = 0.83 AUC (2701 Da) = 0.71 AUC (2094 Da) = 0.70 (P < 0.05) Sn = 95.0%, Sp = 97.1%	Fan *et al*[30], 2013
50 decision trees, 28 masses Three peaks at 3946, 3503 and 15958 Da	Protein Chip SELDI-TOF-MS,	T: GC = 41/HC = 49 V: GC = 28; GC Ⅰ = 9/HC = 30	Sn = 100%, Sp = 96.7% For stage Ⅰ Sn = 89.9% (P = NA) Sn = 92.8%, Sp = 86.7% For stage Ⅰ Sn = 89.9% (P = NA)	Ebert *et al*[24], 2004

[1]Diagnostic values listed for validation set, if not otherwise stated. A2M: Alfa 2 macroglobulin; Apo: Apolipoprotein; AUC: Area under the curve; BGD: Benign gastric diseases; CRP: C reactive protein; DD: D-dimer; ENOSF1: Isoform 2 of mitochondrial enolase superfamily member 1; EGFR: Epidermal growth factor receptor; GA: Gastric adenoma; GC (Ⅰ-Ⅳ): Gastric cancer (TNM stages); HC: Healthy control; HPLC: High performance liquid chromatography; IL-6: Interleukin 6; LC: Liquid chromatography; MALDI-TOF-MS: Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; MB-WCX: Magnetic bead based weak cation-exchange chromatography; NA: Not available; PAI1: Plasminogen activator inhibitor 1; proApo: Proapolopoprotein; RANTES: Regulated upon activation, normally T-expressed and presumably secreted; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry; SERPINA1: Serpin peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin), member 1; Sn: Sensitivity; Sp: Specificity; T: Training set; TTR: Transthyretrin; V: Validation set; VN: Vitronectin.

and 93.6% specificity. In another study, Ahn *et al*[35] constructed a 29-plex array platform based on antibodies against 11 proteins discovered using proteomic approaches and 18 known cancer-associated proteins, and used it to examine serum from 120 GC patients and 120 non-cancerous individuals including 98 gastritis or ulcer patients. They used multivariate classification analysis including 11 analytes (listed in Table 1) that differed between the above-mentioned groups (P value < 0.001). They obtained an accuracy > 85% in an independent validation sample set (95 GC and 51 controls).

By evaluating the known individual serum proteins identified using proteomic approaches from the cancer biomarker perspective, the complexity of the plasma proteome has to be taken into account; it has a wide dynamic range covering 10 orders of magnitude starting from albumins as the most abundant proteins and ending with cytokines and interleukins[36]. Some groups have tried to reduce the plasma proteome complexity by depleting highly abundant protein fractions using different means; however, the results obtained are rather ambiguous. For example, to focus on lower-abundance proteins that might be relevant to cancer, Liu *et al*[37] depleted serum of predominant protein fractions and compared GC and healthy donor specimens using 2D-DIGE followed by MS. They detected 12 differentially expressed proteins including plasminogen, apolipoprotein A-Ⅳ, kininogen-1, clusterin and complement component C4A. Chong *et al*[38] used a combination of proteomic techniques that included highly abundant protein removal and found that plasma protein C9 was significantly increased in GC patients compared with

the healthy donor group. Increased C9 levels have also been reported in serum samples from patients with acute leukemia and sarcoma as well as autoimmune diseases[38]. Ebert *et al*[39] used MALDI-TOF-MS for screening whole serum samples from 14 GC patients and 14 healthy individuals and found that a peptide fragment increased in cancer patients' serum; the peptide was later identified as fibrinopeptide A (FpA). The authors confirmed its level in serum using ELISA in a larger cohort of GC patients (n = 99), high-risk individuals (n = 13) and controls (n = 111), and they observed increased levels in cancer patients and high-risk individuals compared to normal controls. FpA is a blood coagulation protein that is also reported to be a putative biomarker for GC staging[32,40]. The above-mentioned apolipoprotein C-Ⅰ together with C-Ⅲ have been previously reported as diagnostic biomarkers for GC, and the analysis of serum from 103 GC patients and 54 cancer free controls showed decreased expression in the cancer group versus the control group; these results were confirmed using ELISA. The level of apolipoproteins in blood has been reported to be a potential biomarker for various cancers[41]. Yang *et al*[34] identified two peptides that were later characterized as fragments of SERPINA1, an inflammation acute phase protein, and ENOSF1 as the most significantly increased peptides in GC patients. Generally, most proteins mentioned above represent highly abundant plasma proteins and their roles as GC-specific diagnostic markers have to be interpreted with caution, because they are known to be part of a blood coagulation system or represent acute phase inflammatory proteins and they have been reported to be associated with other types of cancer. Evidence from a study using a mouse model of breast cancer showed that the host cell and tumor microenvironment-derived protein signature in plasma differs from the signature associated with inflammatory conditions that are not related to cancer, and therefore could be used for early stage cancer detection[42].

Some studies are focused on posttranslational modification of the serum proteome, such as protein glycosylation[43-47], because it is known that alterations in protein glycosylation are a common feature of tumor cells. Bones *et al*[43], using a combination of glycomic techniques and 2D-DIGE, demonstrated an increased level of sialyl Lewis X epitopes that are presented on triantennary glycans in serum from 80 GC patients compared with 10 patients who had benign gastric diseases and 20 healthy donors, and core fucosylated biantennary agalactosyl glycans were present on extracted immunoglobulin G molecules that were associated with increased TNM stage. Ozcan *et al*[45], by analyzing serum N-glycan profiles using MALDI-TOF-MS, identified 19 glycans that were differentially expressed among patients with GC, non-atrophic gastritis and duodenal ulcers. The glycan profile of the duodenal ulcer group was similar to that in the GC group. In another study, the serum immunoglobulin G glycosylation profile was analyzed using Nano-LC-MS, and eight glycans that can distinguish GC from non-atrophic gastritis, eight glycans that differed between GC and duodenal ulcer and three glycans that differentiated between the non-atrophic gastritis and duodenal ulcer groups were identified[47]. Roy *et al*[46] used an on-chip lectin microarray-based glycomic approach to analyze tissue and serum samples from patients with GC, chronic gastritis and healthy individuals. They showed that the glycoprofile obtained from the tissue samples deviated more than that from the serum samples. It is likely that the altered glycan profile in serum from cancer patients is related to the inflammatory processes and the host defense response mechanisms during carcinogenesis in general[43,45,47].

Although highly promising proteomic diagnostic biomarkers have been identified, especially for early GC diagnosis, there are currently no proteomic-based serum biomarker tests available for clinical application. It has become apparent that large-scale validation studies are critical to evaluate the accumulated proteomic data. Currently, the field of proteomic techniques is rapidly evolving, and continuously-improving technical performance provides constant and reliable high throughput analysis and increasing technical sensitivity for low concentration plasma protein measurements[36,48].

CANCER-ASSOCIATED AUTOANTIBODIES

The human immune system senses the presence of cancer before manifestation of the disease[49]. High-titer IgG class autoantibodies against specific tumor associated antigens (TAAs) have been found in patients' blood even up to five years before clinical diagnosis, thus demonstrating their potential for the detection of early stage cancer[50-52]. In addition, autoantibodies have other promising biomarker qualities: they are found in all tumor types that have been analyzed so far[53,54] and they are highly stable, antigen specific. Unlike the known GC biomarkers such as pepsinogens, CEA and CA19-9, autoantibodies are qualitative, not quantitative, biomarkers. Testing autoantibody reactivity against panels of TAAs using multiplex immunoassays has been shown to be feasible[55] and this aspect might substantially foster their transition from experimental to clinical medicine.

Accumulating evidence has shown that any individual cancer-associated autoantibody biomarker has a limited diagnostic value. Autoantibody repertoires in cancer patients are diverse, and the frequency of antibodies against any particular antigen typically ranges from 1%-15%[56-58]. Among the most studied individual markers in GC, there are autoantibodies against well-known TAAs such as p53 (*e.g.*, 13 studies summarized by Werner *et al*[58] report a biomarker sensitivity range of 8.1-32.1% and specificity range of

Table 2 Autoantibody signatures with diagnostic value for gastric cancer

Biomarker signature description	Technology	Study design	Sample size (GC/controls)	Diagnostic value	Ref.
2 TAAs – p62, Koc	ELISA	GC *vs* HC	135/82	Sn = 19.3%, Sp = 97.6%, $P < 0.01$	Zhang *et al*[62], 2001
3 TSAs - IQGAP3, KRT23 and REG3A	PARSE assay	GC *vs* HC (age and sex matched)	48/46	Sn = 22.9%, Sp = 100%, $P < 0.001$	Xu *et al*[149], 2012
3 TAAs – p16, p53, c-myc	ELISA	GC *vs* HC	74/82	Sn = 21.6%, Sp = 97.6%; $P < 0.001$	Looi *et al*[150], 2006
7 TAAs - p53, C-myc, p16, IMP1, Koc, p62 and Survivin	ELISA	Cardia GC *vs* HC	88/140	AUC = 0.73, Sn = 64%, Sp = 87%, $P < 0.001$	Zhou *et al*[68], 2015
7 TAAs - C-myc, Cyclin B1, IMP1, Koc, P53, p62 and Survivin	ELISA, fixed cut-off	GC *vs* HC	91/346	Sn = 52.7%, Sp = 89.9%, $P < 0.01$	Zhang *et al*[63], 2003
	ELISA, individual cut-off (recursive partitioning)	GC *vs* HC	91/346	Sn = 98.9%, Sp = 93.1%, $P < 0.001$	Koziol *et al*[151], 2003
45 T7 phage-displayed TAA clones (including NY-ESO-1, DDX53, MAGE antigens *etc*.)	T7 phage displayed TAA microarray	GC *vs* HC (age and sex matched)	T:100/100 V:235/213	AUC = 0.79, Sn = 59%, Sp = 90%, $P < 0.001$	Zayakin *et al*[56], 2013
		GC *vs* gastritis	235/100	AUC = 0.64, Sn = 58.7%, Sp = 55%, $P < 0.001$	
		GC *vs* gastric ulcer	235/54	AUC = 0.76, Sn = 58.7%, Sp = 81.5%, $P < 0.001$	

AUC: Area under the curve; GC: Gastric cancer; HC: Healthy controls; ND: Not determined; Sn: Sensitivity; Sp: Specificity; TAA: Tumor associated antigen; TSA: Tumor specific antigen; T: Training; V: Validation.

95.25%-100%), NY-ESO-1[59,60], MUC1[61], Koc, p62[62], C-myc and Survivin[63,64] and others[58].

The development of high-throughput proteomic techniques, such as various native and recombinant protein microarrays and bead-based technologies (reviewed by Meistere *et al*[65]), has enabled the simultaneous detection of autoantibodies against many different TAAs. This has allowed systematic analysis and comparison of the heterogeneous repertoires of circulating autoantibodies within large patient cohorts, which has resulted in selection for cancer-associated biomarker signatures and discarding of those that are induced by other immune processes such as tissue damage, viral infections or possible autoimmune conditions[66,67]. To the best of our knowledge, seven studies have been published on the diagnostic values of different GC-associated autoantibody biomarker combinations (overviewed in Table 2). Within these studies, the identified biomarker signatures could discriminate GC from healthy controls with relatively high specificity (ranging from 87-100%) but with variable sensitivity (19.3%-98.9%). AUC was reported in only two studies: Zhou *et al*[68] showed that autoantibody reactivity against seven known TAAs was able to distinguish between patients with cardia GC from healthy controls with an AUC of 0.73, while Zayakin *et al*[56] reported that 45 GC-associated autoantibody classifiers distinguished GC (all stages with similar sensitivity) from healthy controls with an AUC of 0.79. However, these studies vary greatly in regard to various important aspects, such as the multiplexing level (2-45 autoantibodies), the method used for autoantibody detection, definition of appropriate control group(s), and approaches used for data normalization and cut-off definition. Altogether, these issues may greatly hamper the introduction of the identified biomarkers into clinical practice.

The most relevant biomarkers for early GC diagnosis would be those capable of detecting cancer in high-risk individuals. Only some studies have addressed the GC-associated autoantibody repertoire overlap with that found in patients with benign gastric lesions. For example, a study by Zayakin *et al*[56] found that, within the diagnostic 45-autoantibody signature, the identified biomarker pattern was partially shared between GC and gastritis patients, and was not found in patients with peptic ulcer and healthy controls. Two smaller studies addressed the p53 autoantibody specificity regarding benign gastric diseases and in both cases it was shown that this biomarker specifically detect GC in approximately 32% of the cases and that it is not found in the control patients[69,70]. Another issue is that GC-associated serologically active antigens have been shown to elicit B cell responses in variety of other malignancies[71]. The overlap of the identified GC-associated autoantibody signatures with those found in patients with other (gastrointestinal) cancer types has been addressed only partially and remains to be systematically analyzed within further studies to ascertain their clinical value.

In summary, cancer-associated autoantibody biomarkers have been shown to have high specificity, but moderate sensitivity, which would hinder their use in clinical practice for population-based screening. The limitations of the autoantibody biomarker sensitivity from the biological point of view are currently unknown. In a previous study, we analyzed autoantibody responses against 45 TAAs in 235 GC patients and found no serum-reactivity in 41% of the patients[56]. We then performed extensive screening of cDNA expression libraries with serum samples that did not react against the 45 TAA panel. The screening results showed that up to 10% of the GC patients either generally do not mount an antibody response against tumor antigens or did not have detectable autoantibody levels at the given time

point (unpublished results), thus demonstrating the biological limits for the sensitivity of autoantibody-based diagnostic assays. In addition, heterogeneity of TAA repertoires between cancer patients is high, and each individual autoantibody biomarker generally has a low frequency of detection. Thus, currently-published studies are most likely statistically underpowered. Rare cancer-specific autoantibodies that individually do not reach statistical significance, but are incorporated into the diagnostic biomarker panels, lead to the low reproducibility of initially-obtained results and this lowers the diagnostic value of a diagnostic autoantibody signature, which may be improved by analyzing the proposed biomarker combinations within cohorts with sufficient statistical power.

However, autoantibodies may be important players in the stratification of risk group patients. One of their strengths over other biomarker classes is that the adaptive immune system senses the tumor development early on[49] and can mount high titer antibody responses even to minute amounts of antigen while the presence of other biomarkers (*e.g.*, circulating tumor cells, protein biomarkers, cancer exosomes, cell-free nucleic acids) is gradually increasing in circulation during the progression of cancer. Moreover, the autoantibody repertoires elicited by GC have not been previously analyzed in the context of IgG subclasses. This may be an important aspect because each of the IgG1-4 subclasses have different affinities for activating and inhibiting Fcγ receptors, which eventually has an impact on the activating/inhibitory balance of the infiltrating immune effector cells. This may result in either host-protective or tumor-promoting immune responses, and thus the diagnostic value could be assigned to the specific IgG subclass itself and not only to the antigen specificity of total IgG, as was shown for melanoma[72]. In addition, the analyses of a TAA-specific secreted IgA repertoire might reveal possible novel biomarker candidates because mucosal linings are known to produce more IgA than all other types of antibodies combined.

CELL-FREE NUCLEIC ACIDS

Although the presence of cell-free nucleic acid (cfNA) in human blood was first described by Mandel and Métais[73] in 1948, researchers only began to realize the clinical significance of this finding half a century later[74]. During the past decade, the idea that cfNAs could serve as blood-based biomarkers of cancer has attracted increasing attention. cfNAs may serve as a "liquid biopsy" of cancer reflecting the genetic make-up of tumors, thus enabling detection of drug targets and tracking evolving genetic alterations throughout the course of the disease. Numerous studies have investigated the diagnostic and prognostic potential of total cfDNA levels, gene copy number, DNA integrity, cancer-associated DNA methylation markers or somatic mutations and expression levels of mRNAs, miRNAs and other non-coding RNAs in the blood of cancer patients.

cfNAs can be released into the circulation *via* various forms of cell death such as apoptosis, necrosis, autophagy and necroptosis[74-76] or actively secreted by packaging into extracellular vesicles (EVs)[77-80]. Most of the cfDNA is fragmented and the size distribution of the fragments varies from 150-350 bp to > 10000 bp[81]. The shorter fragments correspond to the mono- and dinucleosomal DNA fragments released from apoptotic cells, while the larger fragments are likely to be released from necrotic cells[81]. Increased cfDNA integrity (*i.e.*, higher ratio of longer to shorter DNA fragments), presumably reflecting an increased rate of necrotic cell death in cancer, has been found in several types of cancer and has been shown to have a diagnostic relevance[82-84]. However, the fraction of tumor-derived DNA has been shown to vary from only 3% to as much as 93% of total cfDNA in different patients[81] and the cellular source of cfDNA is still controversial.

Circulating cfRNA, in particular miRNA, has been found to be remarkably resistant to endogenous and exogenous RNase activity, extreme pH conditions and freeze-thaw cycles[85]. This suggests that cfRNA may be protected from degradation by packaging into various EVs, including exosomes, microvesicles and apoptotic bodies. Studies evaluating the proportion of vesicle-enclosed and vesicle-free miRNA in human plasma, however, have come to controversial conclusions: several studies have showed that the majority of circulating miRNAs are concentrated in exosomes and exosome isolation improves the sensitivity and consistency of miRNA analysis in biofluids[86,87], while other studies showed that only a few miRNAs are enclosed into exosomes[88] and, on average, there is less than one molecule of a given miRNA per exosome[89]. Currently, the reason for such a discrepancy is unclear and more detailed studies on the content, localization and stoichiometry of various RNA species in distinct EV subtypes are required.

Total cfDNA level

Several studies have reported increased levels of total cfDNA in plasma of GC patients compared with healthy controls[90-93] (Table 3). The cfDNA levels could distinguish between GC and control plasma with an AUC varying from 0.75[90] to 0.991[92]. Because the measurement of cfDNA levels does not require any *a priori* knowledge of genetic alterations in the tumor tissue, such an approach could be highly relevant to the development of non-invasive assays for the early detection of GC. However, the size of patient cohorts was relatively small in all of these studies, and therefore validation of the findings in large, well characterized cohorts is required to draw conclusions about clinical utility of cfDNA levels. In addition,

Table 3 Cell-free DNA as biomarkers for detection of gastric cancer

Candidate biomarkers	Sample size and type	Method/technology	Diagnostic value/outcome	Ref.
Total cell-free DNA level				
β-actin (total cf DNA level)	GC = 53, HC = 21, plasma	qPCR	AUC = 0.75, $P < 0.0001$	Sai *et al*[90], 2007
DNA integrity		qPCR (ratio of long *vs* short b-actin amplicons)	No significant difference between GC and HC	
Alu DNA sequences	GC = 54, HC = 59; plasma	Alu81-qPCR	AUC = 0.784, Sn = 75%, Sp = 63%	Park *et al*[91], 2012
Total cfDNA level	Early GC = 16; advanced GC = 14; HC = 34; plasma	Measurement of cfDNA concentration	AUC = 0.991, Sn = 96.67%, Sp = 94.11% for GC *vs* HC	Kim *et al*[92], 2014
Gene amplification				
MYC gene copy number (*MYC/GAPDH* ratio)	GC = 57, HC = 39; tissues and plasma	qPCR	AUC = 0.816; strong positive correlation between MYC levels in GC tissues and plasma ($r = 0.342$; $P = 0.009$)	Park *et al*[99], 2009
HER2 gene copy number (*HER2/RPPH1* ratio)	Discovery: GC = 52 (pre and post-operative treatment), HC = 40; plasma and tissues	qPCR	AUC = 0.746, Sn = 53.9%, Sp = 96.7%; Positive correlation between GC tissues and plasma ($r = 0.424$; $P = 0.00721$); decrease in post-treatment plasma in HER2 + GC cases	Shoda *et al*[100], 2014
	Validation: GC = 25 plasma		Sn = 66.7%, Sp = 100%	
DNA methylation markers				
RPRM (Reprimo)	GC = 43, HC = 31; GC tissues and plasma	MSP	95.3% GC, 9.7% HC, $P < 0.00001$; Strong correlation between methyl status in tissues and plasma	Bernal *et al*[107], 2008
RUNX3	GC (preoperative) = 65, GC (postoperative) = 43, HC = 50, tissues and serum	qMSP	AUC = 0.8651, Sn = 95.5%, Sp = 62.5%; decrease after surgical resection	Sakakura *et al*[152], 2009
KCNA4 + CYP26B1	GC = 46, GPL = 46, HC = 30; serum	Discovery: Methylation microarray in tissues; Testing: MSP	AUC = 0.917, Sn = 91.3%, Sp = 92.1%	Zheng *et al*[109], 2011
SLC19A3	Discovery: GC = 45, HC = 60; plasma	MSRED-qPCR	Increased in GC, $P < 0.0001$	Ng *et al*[153], 2011
	Validation: GC = 20, HC = 20		AUC = 0.82, Sn = 85%, Sp = 85%	
FAM5C + MYLK	GC = 58, GPL = 46, HC = 30; serum	Discovery: MeDIP in cell lines; Testing: MSP	AUC = 0.838, Sn = 77.6%, Sp = 90% for GC *vs* HC; Sn = 30.4% for GPL *vs* HC; decrease after surgical resection	Chen *et al*[154], 2012
XAF1	GC = 202, HC = 88, tumor tissues and serum	qMSP	AUC = 0.909, $P < 0.0001$; 83.9% concordance between GC tissues and serum	Ling *et al*[108], 2013

AUC: Area under the curve; GC: Gastric cancer; GPL: Gastric precancerous lesions; HC: Healthy controls; MeDIP: Methylated DNA immunoprecipitation; MSP: Methylation-specific PCR; MSRED-qPCR: Methylation-sensitive restriction enzyme digestion and real-time quantitative PCR; Sn: Sensitivity; Sp: Specificity.

elevated cfDNA levels have also been detected in patients with inflammatory diseases[94], infections[95] and cardiovascular disorders[96] and in healthy individuals after exercise[97], thus indicating that this phenomenon is not strictly cancer-specific. Similarly, a recent study by Hamakawa *et al*[98] demonstrated that the quantity of DNA fragments harboring cancer-specific somatic mutations in *TP53* gene (circulating tumor DNA, ctDNA) did not correlate with the level of total plasma cfDNA, and only ctDNA showed a good correlation with the GC disease status.

Cancer-specific gene amplification

More specific approaches for measuring total cfDNA levels could be the assessment of cancer-specific genetic alterations in the circulating cfDNA. Several studies have used qPCR to quantify the copy number of genes known to be amplified in GC tissues, such as *MYC*[99] and *HER2*[100,101], in cell-free plasma from GC patients (Table 3). An increased *MYC/GAPDH* ratio in plasma significantly correlated with that in the GC tissues and could distinguish between GC patients and healthy controls with an AUC of 0.816[99]. Similarly, the *HER2* level showed a high correlation in plasma and GC tissues, when quantified using qPCR, and had an AUC of 0.746 for detecting GC[100]. Meanwhile, Lee *et al*[101] reported that the *HER2* copy number in tumor tissues determined by FISH was not significantly associated with the plasma *HER2* level, thus calling into question how well ctDNA levels reflect gene copy numbers in the tumor tissue. The diagnostic usefulness of such tests is limited to detecting GC in patients who harbor the respective genetic amplifications, and therefore they are unlikely to be widely implemented in routine diagnostic examinations. However, they might prove to be highly relevant for detecting the presence or loss of therapeutic targets, and for monitoring treatment efficacy and the course of the disease. Further studies are needed to assess to what extent the ctDNA levels reflect the intratumoral heterogeneity and what factors affect the stability and half-life of the DNA fragments in the plasma.

DNA methylation markers

Several other studies have explored the possibility of detecting cancer-associated hypermethylated DNA fragments in the cfDNA of cancer patients. Methylation markers in the bloodstream were first discovered in breast and lung cancer patients in 1999[102,103]. Lee *et al*[104] demonstrated, for the first time, the feasibility of detecting aberrant methylation in serum from GC patients. This study reported that promoter region hypermethylation of genes encoding DAP-kinase, E-cadherin, GSTP1, p15 and p16 was detected in serum of 48.1%, 57.4%, 14.8%, 55.6% and 51.9% of GC patients, respectively. Subsequently, multiple studies showed hypermethylated genes in the plasma or serum of GC patients. These studies have been systematically summarized in recent reviews by Tsujiura *et al*[105] and Toiyama *et al*[106] and examples of key studies are given in Table 3. Hypermethylated genes showing the highest diagnostic value for detecting GC include *RPRM*[107], *XAF1*[108] and a combination of *KCNA4* and *CYP26B1*[109]. *RPRM* encodes Reprimo, a TP53-dependent cell cycle regulator, and is frequently silenced in GC *via* methylation of its promoter[110]. Bernal *et al*[107] reported that methylated *RPRM* was detected in plasma from 95.3% of GC patients but in only 9.7% of healthy controls, thus yielding a sensitivity of 95.3% and specificity of 90.3%. *XAF1*, a negative regulator of apoptosis inhibitor, has been shown to be downregulated by hypermethylation in cancer tissues of over 83% of GC patients and the agreement between the methylation status in tumor tissues and corresponding serum was 83.9%. Methylated *XAF1* promoter fragments were detected in the serum from 141 out of 202 GC patients, while all 88 cancer-free controls were negative (AUC, 0.909; 95%CI: 0.875-0.942, $P < 0.0001$)[108]. Zheng *et al*[109] used methylation CpG island microarray technology to search for hypermethylated genes in GC tissues and then selected five candidate genes in the serum of 46 GC patients, 46 patients with precancerous lesions and 30 healthy controls. A combination of two methylation markers, *CYP26B1* and *KCNA4*, could distinguish GC from the control serum with a sensitivity of 91.3%, specificity of 92.1% and AUC of 0.917 (95%CI: 0.858-0.976, $P < 0.001$).

These studies have shown several promising methylation markers that warrant further validation in independent cohorts of patients to establish which of the individual markers or combination of markers has the highest diagnostic value. There are also several technical issues that have to be resolved before these assays could be used in a clinical setting. Most of the studies are based on the treatment of DNA with sodium bisulfite, which converts unmethylated cytosine residues to uracil but leaves methylated cytosines unaffected. The modified DNA is analyzed by methylation-specific PCR (MSP) or DNA sequencing. However, these techniques are prone to false-positive results arising mostly from incomplete conversion of unmethylated cytosine residues to uracil[111,112]. Recently, several quantitative techniques for methylation analysis, such as MS-HRM, SMART-MSP, methyl-BEAMing and bisulfite pyrosequencing, have been established[112-114], but their performance in a clinical setting still needs to be validated.

Cell-free RNAs

In 2008, Mitchell *et al*[85] used a mouse model to demonstrate that miRNAs originating from human prostate cancer xenografts enter the blood circulation, thus providing proof of principle that cancer cells release miRNAs that can be detected in the blood. Chen *et al*[115] reported results obtained by deep sequencing of serum miRNAs in patients with diabetes, lung and colorectal cancer and healthy individuals. This study revealed that serum from patients had distinct patterns of disease-specific miRNAs that were absent in the healthy controls and suggested that several diseases may leave specific miRNA-fingerprints in the blood of patients. Recently, more than 20 studies[116] have explored the usefulness of circulating miRNAs for detecting GC. Examples of key studies are given in Table 4. Most of these studies were focused on candidate miRNAs that were selected from previous analysis of GC tissues, while others used a hypothesis-free approach, where miRNA profiling is performed in a discovery sample set using high throughput techniques such as TaqMan arrays, microarrays or deep sequencing, and the diagnostic value of the selected candidate miRNAs is then determined using qRT-PCR in an independent validation set.

Tsujiura *et al*[117] for the first time demonstrated the usefulness of circulating miRNAs for diagnosing and monitoring GC. The levels of five GC-associated miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b and let-7a) were studied in plasma from GC patients and the results showed that the former four miRNAs were present at significantly higher levels while let-7a was decreased in the plasma from GC patients compared to the controls, and the miR-106a/let-7a ratio could distinguish between patients and controls with an AUC of 0.879. Although the authors found relatively good correlation between the miRNA expression levels in the blood and tumor tissue, several subsequent studies showed that only a subset of miRNAs that are highly expressed in tumors show elevated levels in serum or plasma, while other miRNA species are selectively released or retained by the cell[118]. The same group then compared miRNA profiles in pre- and post-operative plasma samples from GC patients using microarray analysis and identified a list of miRNAs that were markedly decreased in post-operative plasma and therefore are likely to be associated with the presence of cancer[119]. Two candidate miRNAs, miR-451 and miR-486, were tested in a cohort of 56 GC patients and 30 healthy controls, and the ROC curve analyses

Table 4 Cell-free RNAs as biomarkers for detection of gastric cancer

Candidate biomarkers	Sample size and type	Method/technology	Diagnostic value/outcome	Ref.
Circulating cell-free miRNAs				
miR-106a/let-7a ratio	GC = 69, HC = 30; plasma	qRT-PCR	AUC = 0.879, Sn = 85.5%, Sp = 80%	Tsujiura *et al*[117], 2010
5-miRNA signature: miR-1, miR-20a, miR-27a, miR-34, miR-423-5p	Discovery: GC = 20, HC = 20; Validation: GC = 142, HC = 105; Serum	Discovery: Solexa sequencing; Testing: qRT-PCR	AUC = 0.831 (validation set)	Liu *et al*[155], 2011
miR-451	Discovery: pre- and post-operative plasma, GC = 3;	Discovery: microarray	AUC = 0.96, Sn = 96%, Sp = 100%; decreased in 90% of post-operation plasma samples	Konishi *et al*[119], 2012
miR-486	Validation: GC = 56, HC = 30	Testing: qRT-PCR	AUC = 0.92, Sn = 86%, Sp = 97%; decreased in 93% of post-operation plasma samples	
miR-378	Discovery: GC = 7, CRC = 7, HC = 10;	Discovery: microarray	AUC = 0.861, Sn = 87.5%, Sp = 70.73%;	Liu *et al*[122], 2012
	Validation: GC = 40, HC = 41; serum	Testing: qRT-PCR	No significant differences across stages Ⅰ-Ⅳ	
miR-223	Test set: GC = 10, HC = 10;	qRT-PCR	AUC = 0.9089	Li *et al*[121], 2012
miR-21	Validation: GC = 60, HC = 60;		AUC = 0.7944	
miR-218	plasma		AUC = 0.7432	
3 miRNA combined			AUC = 0.9531, Sn = 84.29%, Sp = 92.86% No significant differences across stages Ⅰ-Ⅳ	
3-miRNA signature: miR-221, miR-744, and miR-376c	Discovery: GC = 14, HC = 14; Validation Ⅰ: GC = 68, HC = 68 Validation Ⅱ: DYS = 46, HC = 46 Pre-diagnosis serum samples, GC = 58	Discovery: TaqMan array, validation: qRT-PCR	Sn = 82.4%, Sp = 58.8% (for GC *vs* HC) Sn = 73.3% (for early GC) miR-221 elevated in DYS, no difference from HC for miR-376c and miR-744; Increase during GC development; Sn = 79.3% (for GC 2-5 years before diagnosis)	Song *et al*[124], 2012
miR-106b	Discovery: GC = 30, HC = 30	qRT-PCR	AUC = 0.773 (all in validation set)	Cai *et al*[156], 2013
miR-20a	Validation: GC = 60, HC = 60;		AUC = 0.859	
miR-221	plasma		AUC = 0.796	
miR-223	GC = 50, HC = 47; serum	qRT-PCR	AUC = 0.85, Sn = 81%, Sp = 78%; Increased in advanced stages	Wang *et al*[157], 2014
miR-16			AUC = 0.90, Sn = 79%, Sp = 78%	
miR-100			AUC = 0.71, Sn = 71%, Sp = 58% Increased in advanced stages	
miR-16	Discovery: stage Ⅰ non-cardia GC = 40, HC = HC;	Discovery: TaqMan array, validation: qRT-PCR	AUC = 0.768 (all in validation set)	Zhu *et al*[120], 2014
miR-25			AUC = 0.694	
miR-92a	Validation: stage Ⅰ non-cardia GC = 48, HC = 102		AUC = 0.732	
miR-451			AUC = 0.790	
miR-486-5p			AUC = 0.779	
5 miRNA combined			AUC = 0.812, Sn = 72.9%, Sp = 89.2%; In vitro evidence that miR-16, miR-25 and miR92a but not miR-451 and miR486-5p are secreted from cancer cells	
miR-222	GC = 114, HC = 56; plasma	qRT-PCR	AUC = 0.850, Sn = 66.1%, Sp = 88.3%	Fu *et al*[158], 2014
miR-18a	GC = 82, HC = 65, plasma	qRT-PCR	AUC = 0.907, Sn = 80.5%, Sp = 84.6%; no association with stage	Su *et al*[123], 2014
miR-18a	GC = 104, HC = 65, plasma and GC tissues	qRT-PCR	AUC = 0.8059, Sn = 84.6%, Sp = 69.2% Overexpressed in GC; in vitro evidence that miR-18a is released by cancer cells; decreased in postoperative plasma	Tsujiura *et al*[159], 2015
Circulating cell-free mRNAs and long non-coding RNAs				
hTERT mRNA	GC = 118, CAG = 40, HC = 58; plasma	qRT-PCR	AUC = 0.891, Sn = 66%, Sp = 87%; strong positive correlation with advanced stage of GC	Kang *et al*[125], 2013
MACC1 mRNA	GC = 76, HC = 54, plasma	qRT-PCR	Sn = 68%, Sp = 89%	Burock *et al*[160], 2015
LINC00152	Pre- and post-operative plasma GC = 79, GED = 31, HC = 81	qRT-PCR	AUC = 0.657, Sn = 48.1%, Sp = 85.2%	Li *et al*[131], 2015

AUC: Area under the curve; DYS: Intestinal dysplasia; GC: Gastric cancer; HC: Healthy controls; Sn: Sensitivity; Sp: Specificity.

showed an AUC of 0.96 and 0.92, respectively, thus demonstrating their relevance for diagnosing GC and monitoring the course of the disease. However, Zhu *et al*[120] found that these two miRNAs had a lower diagnostic performance (AUC of 0.790 and 0.779, respectively) for detecting early stage non-cardia GC. Surprisingly, both miRNAs were downregulated in GC tissues compared with adjacent normal tissues[119], and their cellular source and the mechanism of release into the circulation remains unknown[120].

Subsequent studies have resulted in the identification of several individual miRNAs or miRNA signatures

that show significant diagnostic values, with an AUC as high as 0.953[121]. Some of the studies report no significant differences in the miRNA levels across GC stages, thus suggesting that these miRNA biomarkers appear in patients' blood at an early stage of cancer development and could be suitable for the detection of early GC[121-123]. A retrospective study by Song *et al*[124] demonstrated an increasing trend in expression of three serum miRNAs (miR-221, miR-744 and miR-376c) over a 15-year timeframe before GC diagnosis and showed that the 3-miRNA panel could classify serum samples collected 2-5 years before the clinical diagnosis of GC with 79.3% accuracy.

Several other studies have explored the possibility of using circulating mRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) for the detection of GC. Despite the presence of RNases in human blood, all these RNA species turned out to be stable and robustly detectable in plasma or serum samples and some of them have shown a relatively high diagnostic value. For example, Kang *et al*[125] reported that elevated *hTERT* mRNA levels could distinguish between GC and healthy controls, with an AUC of 0.891, sensitivity of 66% and specificity of 87%. LncRNAs and circRNAs are recently-discovered categories of non-coding RNAs that regulate gene expression at the transcriptional and posttranscriptional levels and accumulating evidence suggests that they may play key roles in the development of cancer[126,127]. Several recent studies reported that their expression is deregulated in GC tissues and some can be detected in patients' blood[128-132], and thus, they may represent a novel source for circulating biomarker discovery. However, a deeper understanding in their biology, mode of action and mechanism of release into the circulation is required to evaluate their clinical significance.

However, there is a little overlap among the identified miRNAs in various studies and, with a few exceptions such as miR-223 or miR-18a, most of the results have not been reproduced by other studies to date. One of the main reasons for variability and inconsistency among the findings is the approach used to normalize qRT-PCR data. Currently, there is no consensus on housekeeping genes in serum or plasma that could be used as internal controls for this normalization. Several studies have used U6 snRNA or miR-16 as a normalization control, but other studies have shown large fluctuations in their levels in serum and plasma, and they concluded that these RNAs are not suitable as endogenous controls[133,134]. An alternative approach for controlling the technical variability is based on synthetic spike-ins. In this approach, miRNAs without a sequence homology to human miRNAs, such as cel-miR-39, are spiked into the serum/plasma samples before RNA extraction and amplified together with the target miRNAs. The target miRNA levels are then normalized to the sample volume and spike-ins, but this approach does not control for the preanalytical variability. Hemolysis has been shown to alter miRNA content in plasma. For example, miR-16 and miR-451 have been shown to be released by red blood cells and their levels were proportional to the degree of hemolysis[135]. This suggests that assessing the degree of hemolysis is a crucial step in assays that quantify circulating RNA levels.

OTHER POTENTIAL BIOMARKERS

Circulating tumor cells

Detection of the presence of CTCs in the peripheral blood of cancer patients has a promising clinical value in the predictive and prognostic setting, but currently, it has a rather limited potential for detection of early stage cancer. Accumulating evidence shows variable overall GC detection rates based on CTC isolation and characterization of their mRNA expression (ranging from 9.6%-71.2%). Current results are summarized in recent review by Tsujiura *et al*[105]. Studies have shown that the number of CTCs analyzed in peripheral blood from patients with metastatic gastrointestinal cancer is generally lower (1-2 CTCs/7.5 mL of blood) than that found in other malignancies, such as in patients with metastatic prostate cancer (3-5 CTCs/7.5 mL of blood) or breast cancer (6-7 CTCs/7.5 mL of blood)[136-138]. Although novel approaches for rare CTC detection in a small amount of peripheral blood are emerging, their sensitivity for early stage GC is still limited. For example Kolostova *et al*[139] demonstrated that there are biologically inherent limitations to the CTC-based test application for GC detection.

To date, the CellSearch system (Veridex) is the first and only FDA approved test that has been shown to be useful for detecting CTCs in patients with metastatic breast, prostate or colorectal cancer. It enables the enumeration of CTCs of epithelial origin (CD45-, EpCAM+, and cytokeratins 8, 18+, and/or 19+) in whole blood. The usefulness of the CellSearch system in GC detection has recently been evaluated by Uenosono *et al*[140]. The authors showed that the test could detect stage Ⅰ and Ⅱ GC patients in only in 1.6% (1/64) and 3.9% (1/26) of the cases, respectively (P = 0.0002); however, the data indicated that CTC detection in peripheral blood may be a useful tool for predicting tumor progression, prognosis, and the effect of chemotherapy in patients with GC. Besides the CellSearch system, novel and more sensitive experimental approaches for rare CTC detection are being developed; however, the data on their sensitivity for early stage GC is still limited. For example, Kolostova *et al*[139] used the MetaCell® approach, which is based on physical sorting and cultivation of isolated CTCs, to detect one out of three stage Ⅰ GC and two out of four stage Ⅱ GC cases.

Taken together, although numerous studies have been performed, the research on this type of "liquid biopsy" for GC detection remains in its infancy. Further

studies involving larger patient/control cohorts, a deeper understanding of CTC biology and significance and progress in techniques linked to CTC isolation and characterization could enhance their usefulness as biomarkers in future.

Cancer-derived extracellular vesicles

Cancer-derived EVs are gaining increasing attention in the cancer biomarker field[141]. Currently, they are under intense investigation for their composition, biological functions and distribution, along with their diagnostic and therapeutic potential. Either secreted or shed from cancer cells, they are considered to be a liquid tumor biopsy because they are found in elevated levels in the circulation and they have been shown to carry cancer cell-derived lipids, proteins, mRNAs, non-coding and structural RNAs and even genomic DNA, which at least partially reflect parental cells and represents attractive shuttles for cancer biomarkers[142,143]. Studies from several groups have demonstrated the diagnostic potential of cancer-derived EV for the detection of various cancer types, including but not limited to melanoma, prostate, ovarian and colorectal cancer (reviewed by Zocco *et al*[144]). However, there is little data on circulating GC EVs; to the best of our knowledge, only one study has been published regarding the analyses of circulating EVs in patients with stomach cancer. Baran *et al*[145] attempted to characterize the EVs isolated from platelet-depleted plasma samples from 37 GC patients, compared to those from 10 healthy controls. They demonstrated that GC patients, compared with controls, have: (1) a significantly higher number of total circulating EVs (except for patients with stage I GC) ($P < 0.001$); (2) EVs with significantly higher expression of GC-associated proteins MAGE-1 and Her-2/*neu*+ (only late stage patients analyzed, $n = 13$; $P < 0.05$); and (3) EVs with upregulated CRC6 and downregulated CXCR4 surface expression ($P < 0.05$). However, they made no attempt to set a diagnostic value based on these findings. Considering the current advances in this field, further studies on EVs released in patients with GC are warranted.

CONCLUSION

Over the last decade, considerable effort has been dedicated to discovering various types of cancer-associated molecules in the blood of GC patients. Several of the identified biomarkers have remarkably high sensitivity and specificity that greatly outperform the previously-known GC serum biomarkers such PGs, CA 72-4, CA19-9 and CEA[146,147], and therefore have the potential to complement or replace the existing endoscopy, X-ray or biopsy-based examinations. Each type of biomarker has a different origin, provides various types of information and has their own strengths and weaknesses, thus suggesting different clinical applications. For example, autoantibodies against TAAs are qualitative and highly specific markers for the presence of cancer, and they have been identified in the circulation several years before the clinical manifestation of the cancer. Autoantibodies against TAAs, therefore, seem to be an excellent biomarker for the detection of early-stage cancer. However, there is a subset of GC patients with no humoral immunity against tumor antigens that limits the use of autoantibody-based assays for population-based screening programs. Moreover, antibodies are relatively stable and they may remain in the circulation for several months, even years; therefore, they likely have limited potential for monitoring the disease. However, detection of cancer-specific genetic or epigenetic alterations in the cfNA would provide an excellent tool for monitoring cancer dynamics, while their diagnostic use is limited to those patients who have the respective alterations. In addition, these assays may fail to detect evolving cancer cell clones that have lost the respective marker. Several of the proteomics-based biomarker models have demonstrated high sensitivity and specificity for detecting GC; however, it is not clear if most of these proteins are directly and causally involved in the development of cancer and therefore further mechanistic studies are required to validate them as cancer-associated biomarkers.

We suggest that new bio-fluid testing systems, which will combine various types of biomarkers, will be developed in the future and will allow collection of all the information on the disease status, genetic make-up of the tumor and the status of patients' immune system using a single blood test. However, there are several technical issues that have to be resolved before such a device could meet the regulatory requirements. Thus, the next goal would be to perform a head-to-head comparison of various biomarker models and technological platforms in large, well-characterized cohorts of patients and controls to select the biomarkers with highest clinical relevance. This would require a collaborative effort among the research groups to establish standardized pre-analytical and analytical procedures and guidelines for reporting the results.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Role of *Helicobacter pylori* infection in gastric carcinogenesis: Current knowledge and future directions

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Abstract

Helicobacter pylori (*H. pylori*) plays a role in the pathogenesis of gastric cancer. The outcome of the infection depends on environmental factors and bacterial and host characteristics. Gastric carcinogenesis is a multistep process that is reversible in the early phase of mucosal damage, but the exact point of no return has not been identified. Therefore, two main therapeutic strategies could reduce gastric cancer incidence: (1) eradication of the already present infection; and (2) immunization (prior to or during the course of the infection). The success of a gastric cancer prevention strategy depends on timing because the prevention strategy must be introduced before the point of no return in gastric carcinogenesis. Although the exact point of no return has not been identified, infection should be eradicated before severe atrophy of the gastric mucosa develops. Eradication therapy rates remain suboptimal due to increasing *H. pylori* resistance to antibiotics and patient noncompliance. Vaccination against *H. pylori* would reduce the cost of eradication therapies and lower gastric cancer incidence. A vaccine against *H. pylori* is still a research challenge. An effective vaccine should have an adequate route of delivery, appropriate bacterial antigens and effective and safe adjuvants. Future research should focus on the development of rescue eradication therapy protocols until an efficacious vaccine against the bacterium becomes available.

Key words: *Helicobacter pylori*; Gastric cancer; Vaccine; Eradication therapy; Prevention

Core tip: Two main therapeutic strategies could reduce the incidence of *Helicobacter pylori* (*H. pylori*)-related gastric cancer: eradication of the infection or vaccination. Success of a gastric cancer prevention strategy depends on the eradication of the infection or on vaccination before irreversible mucosal changes (severe atrophy, intestinal metaplasia or dysplasia)

have occurred. Eradication therapy results are sub-optimal due to increased antibiotic resistance in *H. pylori* and patient noncompliance. To improve the rates of eradication, rescue regimens have been developed. Concomitant and sequential protocols seem equally effective rescue strategies. An effective vaccine is not available at present, in spite of enormous effort by different researchers.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral, gram-negative, microaerophilic bacterium that plays an undisputed role in the pathogenesis of gastric and duodenal ulcers, low grade B cell gastric lymphoma (MALT lymphoma) and gastric cancer[1]. In 1982, Warren and Marshall cultivated the bacterium. Their discovery changed the therapeutic algorithm for both peptic ulcer disease and gastric MALT lymphoma. The role of *H. pylori* in gastric carcinogenesis was clarified in 1991 when large epidemiological studies[1,2] reported a higher incidence of gastric cancer in *H. pylori*-infected individuals, which confirmed previously published reports[3-5]. Scientific evidence accumulated, and in 1994, *H. pylori* was named as a human carcinogen by the International Agency for Research on Cancer. The role of the infection in gastric cancer development was further supported by a study by Wang *et al*[6] that included 2722 early gastric cancer patients and 13976 controls. This study demonstrated a higher *H. pylori* prevalence in patients with early gastric cancer than in the control group (87% *vs* 61%, respectively).

Gastric cancer is common; it is the third most common of all cancers among males and the fifth most common among females[7]. The survival rate of advanced gastric cancer patients is very low (< 20%). The incidence of gastric cancer is declining in developed countries but rising in developing countries, and the overall burden of the disease is constantly increasing[7,8].

Distinct patterns of *H. pylori* gastritis are related to different outcomes of the infection. Chronic corpus-predominant and multifocal atrophic gastritis lead to increased risk of gastric cancer formation, while antrum-predominant gastritis leads to the formation of duodenal ulcer[9-11].

The outcome of *H. pylori* infection depends on the characteristics of the bacterium in addition to the characteristics of the host and environmental factors.

PATHOGENESIS OF GASTRIC CANCER

Gastric mucosa colonization

H. pylori infection is, in majority of cases, acquired during childhood. The bacterium has to overcome the gastric acid barrier and enter the mucus layer to complete the process of colonization[12] and to subsequently induce damage to the gastric mucosa. Furthermore, the persistence of the infection is also important, and it reflects the ability of the bacterium to adapt to its environment and to start multiplication[13].

To colonize the gastric mucosa the bacterium uses urease activity, motility and adhesion mechanisms[14].

Urease activity is essential for colonization of the gastric mucosa because in the absence of urea, the bacterium can only survive in a pH range of 4.0-8.0, while in an environment containing urea, it remains viable at a low pH of 2.5. Urease catalyzes the hydrolysis of urea into ammonia and CO_2, leading to the increased pH of the bacterial microenvironment. *H. pylori* urease has a high affinity for urea, which enables the bacteria to utilize the limited amounts of urea that are present in the human stomach[15].

H. pylori flagella-mediated motility is necessary for both colonization of the gastric mucosa and for the persistence of the infection[14]. Expression of two flagellar proteins, FlaA and FlaB, is required for full bacterial motility[16].

Adhesion of *H. pylori* to epithelial cells enables the bacterium to alter host cell function. Adhesion is mediated through outer membrane proteins that act as adhesins, including BabA, SabA, AlpA, AlpB and HopZ.

The interaction between the bacterium and the gastric mucus occurs through contacts between the bacterial outer membrane protein BabA[17,18] and the Lewis^b blood group antigen. BabA is a highly variable protein that is encoded by two genes, *babA1* and *babA2*. The protein encoded by *babA2* is functionally active. A major adhesin of *H. pylori* is SabA (sialic-acid binding adhesin), which interacts with sialylated structures on mucins[14]. The proportion of sialylated structures increases in the gastric mucosa during the course of chronic *H. pylori* infection. SabA also binds to sialylated receptors on neutrophils and induces activation of the neutrophils. AlpA and AlpB are expressed in all bacterial strains and enable binding to host laminin[14,18]. HopZ also plays role in the colonization process[14,19].

H. pylori uses the thioredoxin system[14,20] to induce partial breaks and changes in the polymeric structure of mucus gel. *H. pylori* infection and non-specific mechanisms of inflammation simultaneously reduce the protective capabilities of gastric mucin. One-fifth of the presenting bacteria completely adhere to the gastric surface epithelium, while the remaining bacteria reside in the surface mucus layer[21]. The helical shape of the bacterium facilitates its penetration of gastric mucus[22].

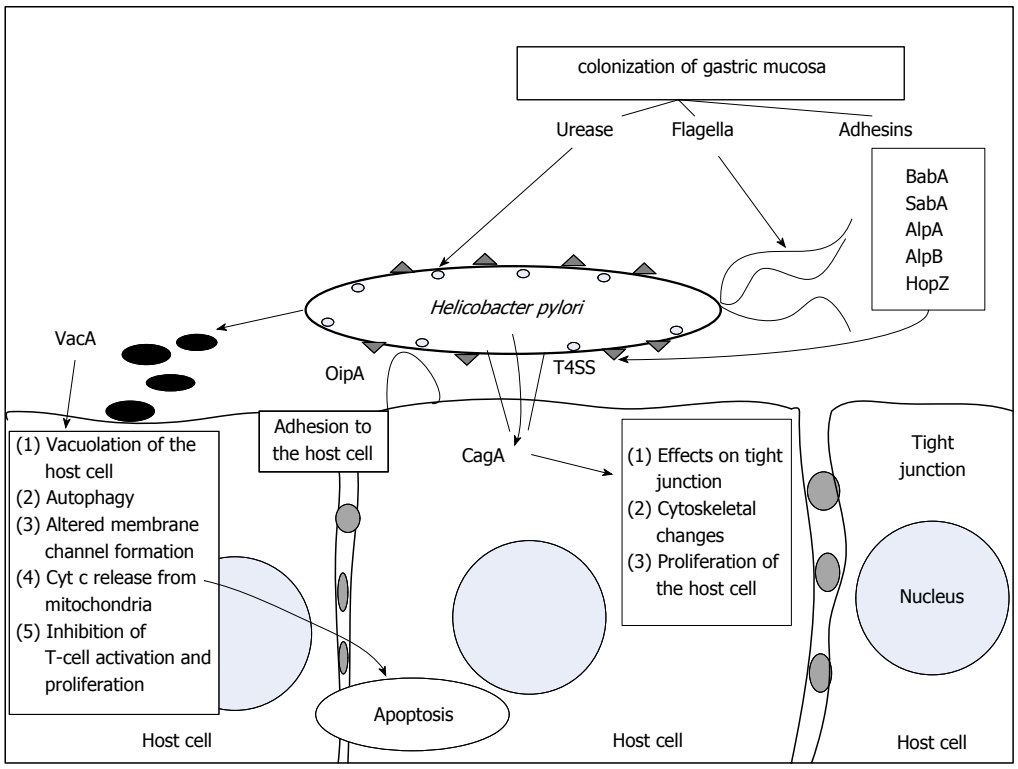

Figure 1 Mechanisms of *Helicobacter pylori* induced gastric mucosa damage.

H. pylori virulence factors

H. pylori virulence depends on the above described and other factors that are responsible for damage to the gastric mucosa (Figure 1). Epidemiological studies have identified six distinct *H. pylori* strains in different geographic regions that are related to different incidences of gastric cancer[23]. These strains are termed hpEastAsia, hpAsia2, hpEurope, hpAfrica1, hpAfrica2 and hpNEAfrica. *H. pylori* produces various virulence factors and has the ability to modulate its reaction to the host immune response and to thereby adapt to individual host conditions[8].

H. pylori is a highly heterogeneous bacterium[24,25]. Virulence factors that contribute to gastric cancer development include the cytotoxin-associated gene (*cagA*) A and CagA protein (CagA), CagL, vacuolating cytotoxin (VacA) and outer inflammatory protein (OipA), while the possible role of the duodenal ulcer-promoting gene (*dup*A) remains unclear[8].

CagA

Two distinct types of *H. pylori* are the CagA-producing (*cagA*-positive) strains and the CagA-nonproducing (*cagA*-negative) strains. In animal models, gastric cancer develops only in animals infected with cagA-positive *H. pylori* strains or when CagA protein is artificially introduced into the host[26,27]. Because in humans, only some individuals infected with *cagA*-positive strains develop gastric cancer, further investigations have focused on *cagA* gene polymorphisms. The number of repeat sequences in the 3′ region of the *cagA* gene differs between *H. pylori* strains[28]. Each repeat region of the CagA protein contains EPIYA motifs, a term used to describe a specific sequence of amino acids (Glu-Pro-Ile-Tyr-Ala). There are two EPIYA motifs in the first repeat region (EPIYA-A and EPIYA-B) and two in the second (EPIYA-C or EPIYA-D) repeat region[24]. In Western-type *H. pylori*, CagA proteins have EPIYA ABC, ABCC or ABCCC repeat regions, while in East Asian-type *H. pylori*, CagA proteins have EPIYA ABD repeats[8,24].

The CagA protein consists of a C-terminal region that contains the EPIYA motifs and an N-terminal region[8]. After adhesion of the bacterium to the host cell, CagA is injected into the host cell *via* the *cag* pathogenicity island (*cag*PAI)-encoded type IV secretion system (T4SS), and electrostatic interactions with phosphatidylserine keep CagA linked to the inner leaflet of the cell membrane[8,29]. In the cytoplasm of the host cell, CagA is phosphorylated at its EPIYA motifs[30]. CagA alters host cell signaling in both a phosphorylation-dependent and a phosphorylation-independent manner[8]. The induction of heme oxygenase 1 reduced CagA phosphorylation in gastric epithelial cells *in vitro,* while *in vivo H.pylori* diminishes heme oxygenase 1 gene expression[8,31].

CagL

Carcinogenic and virulent *H. pylori* strains express CagL, a highly conserved protein component of T4SS that is involved in bacterial attachment to the host

cell and also in the induction of host inflammatory responses and carcinogenesis[29]. CagL induces hypergastrinemia, which is a risk factor for the development of gastric adenocarcinoma[8,30]. Contact between CagL and $\alpha 5\beta 1$ integrin induces IL-8 secretion from the host cell[32].

VacA

VacA induces vacuolization and apoptosis (as a consequence of *cytochrome c* release from mitochondria) of the host cell. It is also responsible for altered membrane-channel formation and the induction of autophagy and altered host immune responses[30,33,34], mainly through the inhibition of T cell activation and proliferation[35]. The *vacA* gene is functional in all *H. pylori* strains and differences in its vacuolating activity have been associated with its gene structure, which varies in the signal (*s1* and *s2*), middle (*m1* and *m2*) and intermediate (*i1* and *i2*) regions[36]. The risk of developing different gastrointestinal pathology is attributable to different combinations of *s, m* and *i* region subtype.

The *s1/m1* strains induce the highest level of cytotoxicity, the *s1/m2* strains induce the lowest, and the *s2/m2* strains have no cytotoxic activity (*s2/m1* strains are rare)[37]. The risk of developing either gastric cancer or peptic ulcer disease is increased in individuals infected with *s1* or *m1 H. pylori* strains compared with individuals infected with *s2* or *m2* strains[8,24]. In East Asia, most *H. pylori* strains are *s1* type, and in these patients, the presence of the *m1* region is related to an increased risk for gastric cancer[24].

The intermediate region of *vacA* is localized between the *s* and *m* regions. Type *i1* is found in all *s1/m1* strains, while all *s2/m2* strains are type *i2*. Strains that are type *s1/m2* can be either type *i1* or *i2*. Strains with the *i1* region are more pathogenic[8]. The type of the *i*-region has a better predictive value than *s* region type in some, but not all populations[38].

The deletion (*d*) region is localized between the *i* and the *m* regions[39]. The *d* region can be type *d1* or *d2*. In patients infected with Western strains, the presence of the *d1* region is a risk factor for gastric mucosal atrophy. In patients infected with the East Asia type of *Helicobacter*, all strains are classified as *s1/i1/d1*[24].

OipA

OipA is a protein that was identified in 2000[24] and is involved in *H. pylori* adhesion to the host cell, induction of the host pro-inflammatory response and the subsequent increase in mucosal interleukin-8 (IL8) levels[40]. Results from animal studies suggest a role for OipA in gastric carcinogenesis[8,26].

CagA, VacA and OipA synthesis is linked. Therefore, almost all *H. pylori* strains produce either all or none of these proteins. East Asian *H. pylori* strains are highly pathogenic and CagA, VacA and OipA-producing. According to the available data, the presence of these three genes is related to gastric cancer and peptic ulcer disease pathogenesis[24].

DupA

Duodenal ulcer-promoting gene (*dup*A) plays a role in T4SS formation and is localized in the plasticity zone. Lu *et al*[41] proposed *dupA* as an *H. pylori* virulence factor that is involved in duodenal ulcer pathogenesis and that confers a protective effect against gastric carcinogenesis[8], but other studies have failed to demonstrate a relationship between this gene and any distinct gastroduodenal pathology, and they have therefore not supported this hypothesis[23,42,43]. These results could be explained by polymorphism of the *dupA* gene[30].

H. PYLORI, GASTRIC CANCER AND GEOGRAPHY-ROLE OF BACTERIAL STRAIN

Multilocus sequence typing of housekeeping genes revealed that there are six distinct *H. pylori* strains (hpEurope, hpEastAsia, hpAsia2, hpAfrica1, hpAfrica2 and hpNEAfrica). These strains are associated with different geographic regions and with the incidence of gastric cancer.

It is probable that housekeeping gene differences are merely markers for virulence factors that affect disease outcome[8,23]. Initially, four main clusters were identified by Falush *et al*[44]. HpEurope isolates were found in Europe and in countries colonized by Europeans, while the majority of isolates from East Asia were the hpEastAsia strain. HpAfrica1 is widely spread, while hpAfrica2 is found exclusively in South Africa. Two clusters were later identified: hpAsia (South and Southeast Asia) and hpNEAfrica (the predominant isolate in Northeast Africa)[8,25].

The geographical distribution of HpEastAsia isolates is concordant with the high incidence of gastric cancer in these areas. On the other hand, in low gastric cancer incidence areas, such as Africa and South Asia, most strains are hpNEAfrica, hpAfrica1, hpAfrica2 or hpAsia2. This is a plausible explanation for both African and Asian enigma. The high incidence of *H. pylori* infection is related to the high incidence of gastric cancer in East Asia, while a low incidence of gastric cancer is observed in populations with a high prevalence of *H. pylori* infection in Africa (the African enigma) and South Asia (the Asian enigma)[8,23].

Changes in the infected mucosa

After infection with *H. pylori*, inflammation and mucosal damage occur in the non-acid secreting gastric antrum. Over time, mucosal damage progresses into the gastric corpus. The atrophic border can be recognized

endoscopically, and the damage progresses more rapidly along the lesser curve than the greater curve, as previously reported[44,45]. Chronic inflammation that is related to *H. pylori* affects cell differentiation and promotes metaplasia[46-48]. As the damage spreads into the corpus, pyloric metaplasia is observed near the atrophic border. Pyloric metaplasia exhibits similar immunohistochemical characteristics to spasmolytic polypeptide/trefoil factor family 2-expressing metaplasia (SPEM), which has been described in animal models of gastric carcinogenesis[49,50] and is probably an important step in gastric cancer formation.

DEVELOPMENT OF GASTRIC CANCER

Gastric adenocarcinoma can originate from both proximal (cardia) and distal (non-cardia) parts of stomach. Proximal and distal gastric cancers have different epidemiological and clinical characteristics[8]. Risk factors for proximal gastric cancer include increased body weight, gastro-esophageal reflux disease and Barrett's esophagus[8,51], while distal gastric cancer risk is increased by the presence of *H. pylori* infection[9-11], family history of gastric cancer, low socioeconomic status, smoking and a diet rich in salty and smoked food with low consumption of fruits and vegetables[52].

According to the Lauren classification, gastric cancer is divided into intestinal and diffuse histological subtypes. The presence of *H. pylori* infection and corpus-predominant gastritis with intestinal metaplasia leads to intestinal-type gastric cancer, whereas diffuse gastric cancer arises from non-atrophic pangastritis[9-11].

Long-lasting precancerous processes result in intestinal-type gastric adenocarcinoma. In 1938, pathologists proposed that the presence of gastric intestinal metaplasia is related to gastric cancer[8]. This model, now known as the Correa cascade, was reintroduced and proposed by Correa *et al*[53] in 1975. The authors updated their model in 1988 and 1992. In the Correa cascade, consecutive histological changes in the gastric mucosa occur, leading to gastric cancer through the following steps: normal gastric mucosa, superficial (non-atrophic) gastritis, multifocal atrophic gastritis, complete (small intestine type) intestinal metaplasia followed by intestinal metaplasia of the incomplete (colonic) type, low-grade dysplasia, high-grade dysplasia and invasive adenocarcinoma[9-11,53]. It is now believed that intestinal metaplasia arises from SPEM and that SPEM may also provide the cells of origin for gastric cancer[50,54]. Intestinal metaplasia is considered by some authors as a surrogate marker for the presence and extent of gastric mucosal atrophy[55-57]. The concept of multifocal atrophic gastritis represents areas of intestinal metaplasia in SPEM-type atrophy damage[58,59]. Nevertheless, the critically important point of no return, up to which gastric cancer prevention is possible and histological changes are reversible, remains unidentified[8].

HOST FACTORS

Apart from the bacterial strain, the characteristics of the host play a role in gastric carcinogenesis. Different host gene polymorphisms have been described, mainly as single nucleotide polymorphisms. These polymorphisms influence host inflammatory immune responses and affect host cell proliferation and mucosal protection. They also exert an effect on the metabolism of carcinogens and antioxidants[8].

STRATEGIES FOR GASTRIC CANCER PREVENTION

There are two main therapeutic strategies that could reduce the incidence of gastric cancer: eradication of an ongoing infection or immunization prior to or during the course of the infection (Figure 2). The success of a gastric cancer prevention strategy depends on its timing because prevention strategies should be introduced before the point of no return in gastric carcinogenesis. Although the exact point of no return has not been identified, infection should be eradicated before severe atrophy of the gastric mucosa develops.

ERADICATION OF *H. PYLORI* INFECTION IN GASTRIC CANCER PREVENTION-CURRENT EVIDENCE

Effects of eradication therapy for *H. pylori* infection on either invasive gastric cancer or premalignant histological lesions of the gastric mucosa have been reported in five randomized control trials (RCT)[60-65].

Gastric cancer incidence was evaluated in a RCT conducted by Wong *et al*[60] that lasted for 7.5 years. Healthy individuals were randomized to receive either eradication therapy or placebo in a region in China with a high gastric cancer incidence. The study results did not demonstrate a decrease in overall gastric cancer incidence, but the results did suggest a protective role for *H. pylori* eradication in subjects without precancerous lesions[8].

The low number of gastric cancer cases[60,63] and the study design (it did not aim to assess gastric cancer incidence as a primary outcome) of other previously published studies[61,62] has led to a lack of scientific evidence for the possible effects of *H. pylori* eradication on cancer occurrence. Evidence in favor of a protective effect of *H. pylori* eradication on gastric cancer formation came from a study by Ma *et al*[64]. They published the results of a RCT after 15 years of follow-up with 2258 *H. pylori* seropositive adults. In this study, participants were selected from the general population and randomly assigned to one of three intervention groups (*H. pylori* eradication, garlic

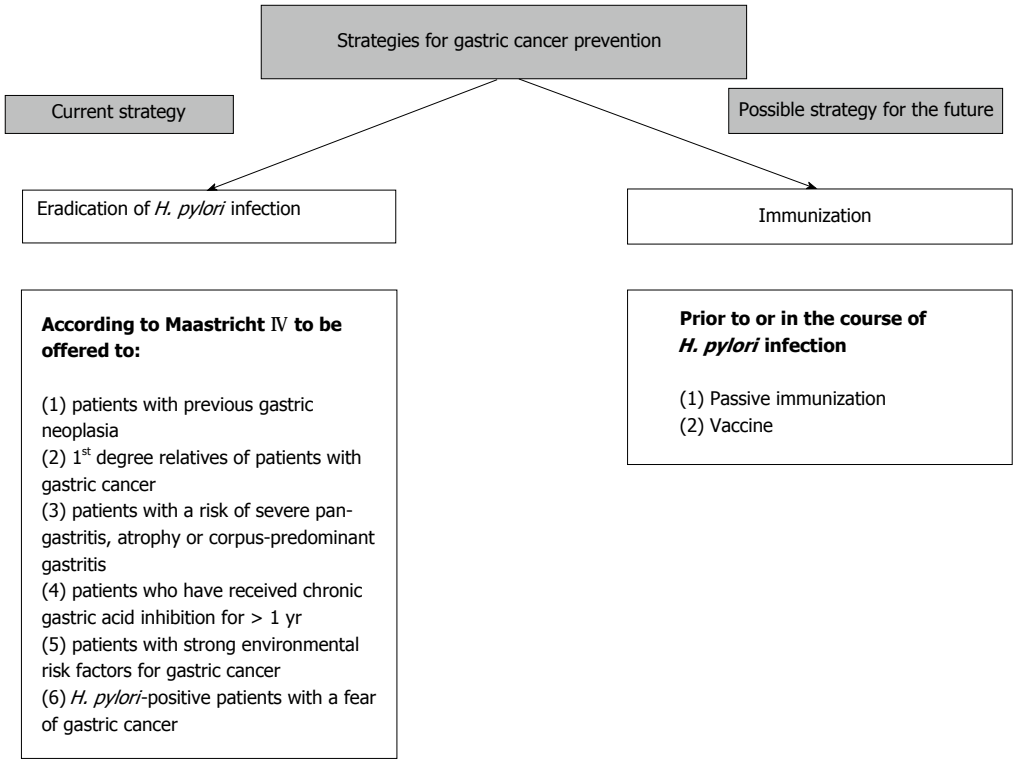

Figure 2 Strategies for gastric cancer prevention. *H. pylori*: *Helicobacter pylori*.

supplements, or supplemental vitamins) or a control group. This study demonstrated reduced gastric cancer incidence in the group of patients treated with eradication therapy for *H. pylori*.

The benefits of mass eradication in populations with high incidence of *H. pylori* was assessed by Lee *et al*[66]. Individuals with positive *H. pylori* urea breath test underwent endoscopic screening and received eradication therapy. The success rate of the eradication therapy was 78.7%, and it led to a decrease in gastric atrophy incidence. However, no significant change in intestinal metaplasia was observed. The incidence of gastric cancer decreased by 25% during the study.

An important study from Uemura *et al*[67] assessed the effect of *H. pylori* eradication on metachronous gastric cancer development in patients who had a previous endoscopic resection of an early gastric carcinoma. *H. pylori*-positive patients who underwent endoscopic resection were randomized to receive either *H. pylori*-treatment or no treatment. After four years of follow up, metachronous cancer was not diagnosed in any of the *H. pylori*-treated patients compared to 9% in the group that received no treatment. These findings were confirmed in a larger study by Fukase *et al*[65], who demonstrated that eradication therapy in patients with previous endoscopic resection of early gastric cancer reduced the risk of metachronous gastric carcinoma by 65%. Current Japanese guidelines reflect an acceptance of the results of this study and suggest *H. pylori* eradication therapy after endoscopic resection of early gastric cancer[68].

Recently published data are available from a retrospective study performed in South Korea[69] that analyzed the relationship between the risk of metachronous gastric cancer in patients who underwent endoscopic resection of early gastric cancer and *H. pylori* eradication therapy. This study confirmed the results of Fukase *et al*[65] and demonstrated that successful *H. pylori* eradication may reduce the occurrence of metachronous gastric cancer.

Recent trial reports[64-66,69] have also provided evidence of a protective effect of *H. pylori* eradication in gastric cancer.

INDICATIONS FOR ERADICATION IN GASTRIC CANCER PREVENTION

According to the Maastricht IV consensus, eradication of *H. pylori* reduces the risk of gastric cancer development. Patients should be treated during the initial phase of the infection, before preneoplastic changes in the gastric mucosa occur. Authors of the Maastricht IV consensus identified individuals with an increased risk for gastric cancer. They suggest that to decrease gastric cancer risk, eradication therapy should be offered to first-degree relatives of family members with a diagnosis of gastric cancer, patients with previous gastric neoplasia who have already been treated by endoscopic or subtotal gastric resection, patients with a risk of severe pan-gastritis, corpus-predominant gastritis or severe atrophy, patients who have received chronic gastric acid inhibition for more

than 1 year, patients with strong environmental risk factors for gastric cancer (heavy smoking or high exposure to dust, coal, quartz, cement and/or work in quarries), and *H. pylori*-positive patients with a fear of gastric cancer[70]. Two other documents, the American College of Gastroenterology guidelines[71] and the Asia Pacific Consensus document[72], have also recommend *H. pylori* eradication in patients with endoscopic resection of early gastric cancer.

Population screening and eradication of *H. pylori* was a matter of scientific debate until recently. Currently, the Maastricht IV consensus encourages[70], while the Asia Pacific consensus strongly recommends, population screening and treatment for *H. pylori* in high-risk regions as a chemo-prophylactic measure for gastric cancer[71]. The rationale behind this is the cost effectiveness of eradication therapy when compared to the cost of treatment of advanced gastric cancer.

TIMELY ERADICATION IS ESSENTIAL FOR GASTRIC CANCER PREVENTION

H. pylori eradication therapy should stop the progression of mucosal damage and reduce gastric cancer risk[73]. Eradication of the infection stops the inflammatory process and promotes healing of gastritis and a resolution of inflammation. *H. pylori* leads to gastric cancer through the Correa cascade; therefore, when severe atrophic damage and intestinal metaplasia occur, eradication cannot reverse mucosal changes.

After eradication therapy, individuals with non-atrophic gastritis have a negligible risk of developing gastric cancer, while individuals with atrophic gastritis have an increased risk. This risk is overall lower in eradicated patients when compared to untreated patients with the same pattern of gastritis. In untreated patients, the risk for gastric cancer increases yearly as the atrophy progresses[73], as demonstrated by Ohata *et al*[74] In a large, longitudinal cohort study on 4655 healthy asymptomatic subjects followed for 7.7 years, the authors aimed to determine the association between *H. pylori* infection and the progression of chronic atrophic gastritis (CAG) with gastric cancer. The authors identified 45 gastric cancer cases, none of which were *H. pylori* negative and CAG negative, during the study period. Development of CAG increased the risk of gastric cancer. Recently published data[75] from the same group confirmed that in subjects with a serologically diagnosed healthy stomach (*H. pylori*-negative/pepsinogen within normal range and therefore CAG-negative), the cancer incidence rate was low (16/100000 person-years). On the other hand, in the individuals with an *H. pylori* infection, they observed progression of chronic gastritis and increased gastric cancer risk. In patients with no atrophy and active inflammation, gastric cancer risk was estimated at 250/100000 person-years, which is comparable to the risk in subjects with CAG. Patients with active inflammation were at risk of diffuse gastric cancer. These results revealed that gastric cancer develops in some patients as a result of the Correa cascade, while in others it can result from a direct carcinogenic pathway based on active inflammation.

Eradication therapy, up to some point, prevents gastric cancer. Data on the possible reversion of atrophic changes in the gastric mucosa is conflicting. A longitudinal cohort study conducted by Yanaoka *et al*[76] with a mean follow up of 9.3 years demonstrated a significant reduction in cancer incidence after eradication in *H. pylori* positive patients with mild atrophic gastritis. The incidence in patients with persistent infection was 111/100000 person-years compared to 69/100000 among patients in whom the infection was eradiated. As expected, cancer incidence rates did not vary significantly (237 *vs* 223) among the patients with severe atrophy. The authors concluded that cancer development after eradication depends on the presence of extensive CAG before eradication and that *H. pylori* eradication is beneficial in subjects with mild CAG. A study by Sakakibara[77], who followed a small group of 8 patients who were surgically treated for gastric cancer for 9 years, suggested a prompt improvement in the atrophy score (reversion of atrophic changes) in the remaining gastric mucosa following eradication therapy. The authors concluded that *H.pylori* eradication improved possible precancerous lesions in the gastric remnant.

Watari *et al*[78], in a prospective study, followed 96 patients for 4 years who exhibited chronic gastritis with or without intestinal metaplasia or gastric intestinal metaplasia with dysplasia and failed to demonstrate a change in intestinal metaplasia score. Nevertheless, the authors reported a change in the intestinal metaplasia phenotype: they followed the expression of several biomarkers related to carcinogenesis and demonstrated regression in TC22-4. TC22 is a neoplastic marker that is expressed exclusively by transformed epithelial cells. Based on this finding, they suggested that the change of phenotype may be an important factor in the reduction of cancer incidence after eradication of *H. pylori*. *H. pylori* eradication prior to development of intestinal metaplasia was beneficial for patients with corpus gastritis. However, eradication in high risk patients (*i.e.*, patients with atrophy with intestinal metaplasia, especially of the incomplete type or with a history of endoscopic treatment for gastric cancer) was not beneficial[79]. Identification of the point of no return for the development of malignancy is an important, but still unanswered, scientific goal.

SUCCESS OF ERADICATION THERAPY

There are two main underlying causes of suboptimal results of eradication therapy: *H. pylori* resistance and patient noncompliance (Figure 3).

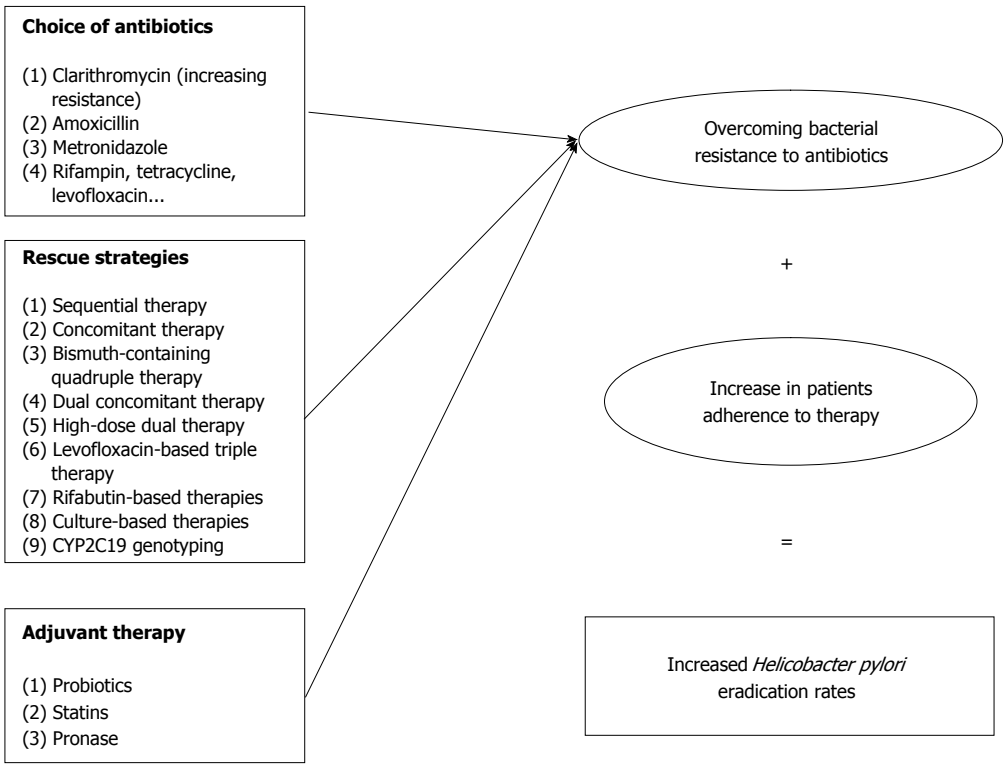

Figure 3 Factors influencing *Helicobacter pylori* eradication rates.

CHOICE OF ANTIBIOTICS IN THE LIGHT OF INCREASING BACTERIAL RESISTANCE

In the first years after the discovery of *H. pylori* and its role in the pathogenesis of major gastric diseases, eradication therapy seemed to be a safe and efficient strategy that would resolve and eradicate peptic ulcer disease, MALT lymphoma and majority of gastric cancer cases.

In 1993, an eradication protocol consisting of two antibiotics (clarithromycin and amoxicillin or metronidazole) and a proton pump inhibitor was proposed[80,81] and confirmed as efficacious in large studies[82,83].

Eradication therapy is effective when the mucosal concentration of the antibiotic is above the minimal bactericidal concentration (MBC) at the site of the infection for a sufficient time, which enables the eradication of all present bacteria[84]. Macrolides (clarithromycin), beta-lactams (amoxicillin), tetracycline, metronidazole, rifampin (rifabutin) and fluoroquinolones (levofloxacin) were identified as antibiotics with the best potential for eradication of the infection. For these drugs, therapeutic doses result in a mucosal concentration above the MBC.

Selected antibiotics need to be absorbed and released in the gastric mucosa over long periods of time. The *in vitro* efficacy of aminoglycosides and bismuth salts is compromised *in vivo* by a poor rate of absorption. In addition, some drugs from the above mentioned groups have displayed disappointing clinical effects, *i.e.*, doxycycline (tetracycline) and ciprofloxacin (fluoroquinolone). There are also other factors that are described in detail in a review by Megraud[85] that should be considered when assessing antibiotic efficacy. These factors include the acidity of the stomach and bacterial resistance. The majority of antibiotics are not active at low pH and are only active in dividing bacteria, making the use of proton pump inhibitor (PPI) mandatory.

Clarithromycin is the basis for *H. pylori* treatment because it has MBC, good mucosal diffusion and is not affected by gastric acidity. The addition of a second antibiotic provides a high and permanent eradication rate. The second antibiotic of choice is either amoxicillin or metronidazole. Triple therapy was a standard treatment until recent years, in which we are facing increased *H. pylori* resistance to clarithromycin that is attributable to the selection of bacteria with point mutations that have occurred during replication[86,87].

The global clarithromycin resistance rate in Europe increased from 9% in 1998[88] to 17.6% in 2008[89]. It has been suggested that clarithromycin resistance is the major cause of eradication treatment failure. Data on clarithromycin resistance was obtained in a study that included 18 European countries. Primary *H. pylori* antibiotic resistance rates were, for adults, 17.5% for clarithromycin, 14.1% for levofloxacin and 34.9% for metronidazole. Higher resistance rates for clarithromycin and levofloxacin are observed in Western/Central and Southern Europe (> 20%) than

in Northern European countries (< 10%). There are also data from individual countries showing a wide range of resistance rates to clarithromycin. Resistance rates range from 0% in India to 49.2% in Spain[90,91]. There is also cross-resistance for all of the other macrolides[85].

Resistance to rifampin, amoxicillin and tetracycline is rare[85]. Resistance to rifampin is observed in patients previously treated for tuberculosis, while resistance to tetracycline has been reported in some[92,93], but not all studies from Korea and Brazil[94,95]. Resistance to metronidazole is not frequently observed and depends on the other drugs used and the length of treatment[85].

The first Maastricht conference proposed a triple treatment including PPI-clarithromycin and amoxicillin or metronidazole[96] that currently has an eradication rate of 70% (the aimed-for eradication rate for any protocol should be over 80%)[97]. Possible explanations for this decrease in efficacy of the standard triple therapy include low compliance, high gastric acidity, high bacterial load, the type of strain and an increase in *H. pylori* resistance to clarithromycin. Maastricht IV therefore suggests that PPI-clarithromycin-containing triple therapy without prior susceptibility testing should not be prescribed in regions with a clarithromycin resistance rate of more than 15%-20%[70].

RESCUE STRATEGIES FOR OVERCOMING BACTERIAL RESISTANCE

Because no new drug has been developed for this indication, a number of studies have focused on the use of different combinations of known antibiotics.

Possible scenarios proposed to overcome the problem of low eradication rates include the administration of sequential or concomitant therapy. Sequential therapy (ST) consists of a PPI and amoxicillin administered for the first five days followed by a PPI and 2 other antibiotics for the following 5 d. This sequential administration weakens the bacterial cell wall in the initial phase and helps to increase eradication rates, even in clarithromycin-resistant strains. Concomitant therapy (CT) regimen consists of all of the medication administered in ST, but given simultaneously. The efficacy of both ST[97,98] and CT[99,100] therapies has been supported in different studies. A recent meta-analysis provided data on the efficacy of concomitant *vs* sequential therapies[101]. The analysis was based on 7 RCTs that included 2412 individuals. The sequential regimen was successful in 83.8% of patients, while concomitant therapy eradicated the infection in 86.1% of patients. The adverse events and adherence to medications were not different between the two regimens.

The idea of a bismuth-containing quadruple therapy was revisited and improved through the single pill concept. Namely, a formulation containing bismuth salts, tetracycline and metronidazole in the same pill was developed[102-104].

Hsu *et al*[105] proposed hybrid (dual concomitant) therapy consisting of dual therapy (PPI and amoxicillin for 7 d) followed by a concomitant quadruple therapy (PPI, amoxicillin, clarithromycin and metronidazole for another 7 d). The eradication rate for this treatment was over 97%.

High-dose dual therapy consists of administration of PPI and amoxicillin three times a day for 2 wk and was initially designed for areas with high resistance to clarithromycin. It provides eradication in 78.4% of patients[106,107] and does so with fewer side effects and better compliance. The authors therefore suggested that larger studies are needed[108].

Levofloxacin-based triple therapy consists of PPI, levofloxacin, and amoxicillin for 10 d, and the eradication rate of levofloxacin-based triple therapy ranges from 74% to 96%[109-111]. This regimen is not recommended as the first line treatment because augmented use of quinolones for respiratory and urogenital infections increased *H. pylori* resistance to these drugs[109]. The resistance to levofloxacin is a consequence of a point mutation in a special region, the so-called quinolone resistance determining region. There is also cross-resistance in all fluoroquinolones[91]. Levofloxacin-based therapy is considered to be an efficient alternative regimen in populations with 15%-20% clarithromycin resistance and quinolone resistance less than 10% and is a second line treatment, according to the Maastricht IV consensus[70]. Selection of quinolone therapy should be based on the results of antibiotics susceptibility tests or geographic resistance patterns due to the rapid increase in the number of resistant strains[91].

Rifabutin-based therapies were introduced as rescue therapies based on the results of *in vitro* studies[112]. A triple regimen includes amoxicillin, PPI and rifabutin, but the optimal duration of treatment is not defined and ranges from 7 to 14 d[113-115]. Myelotoxicity is a rare but significant complication that limits its widespread use[113]. The potential for mycobacterial resistance also limits the use of this regimen, leaving it as valid option only as a rescue treatment.

Culture-based therapies are recommended after the failure of second-line treatments. An antimicrobial susceptibility test is recommended[69], and treatments adjusted to the results achieve more than 90% eradication rate after second-line therapy failure[116]. The test is invasive, expensive and has low sensitivity (less than 60%)[117]. Mixed infections with susceptible and resistant *H. pylori* strains also limit the efficacy of this therapeutic approach[118].

The relevance of CYP2C19 genotyping as a rescue strategy for improvement of eradication rates is based on the fact that the CYP2C19 polymorphism affects the *H. pylori* eradication rate, especially by omeprazole treatment[119]. This strategy is probably plausible in

Table 1 Adherence to eradication therapy protocols in different countries

Ref.	Country	Adherence to treatment
Wu *et al*[130]	Taiwan	98.2% concomitant therapy 95.7% sequential therapy
Greenberg *et al*[131]	Latin America (Chile, Colombia, Costa Rica, Honduras, Nicaragua, and Mexico)	92.2% overall 93.8% concomitant therapy 93% sequential therapy 89.9% standard therapy
McNicholl *et al*[132]	Spain	83% concomitant therapy 82% sequential therapy
Lim *et al*[133]	South Korea	96.2% concomitant therapy 95.3% sequential therapy
Huang *et al*[134]	Taiwan	94% concomitant therapy 95.3% sequential therapy
Hsu *et al*[135]	Taiwan	100% concomitant therapy 98% sequential therapy 99% standard therapy

Asia, where poor metabolizers account for more than 15% of all patients[72,120].

ADJUVANT THERAPY

Adjuvant therapy in *H. pylori* eradication is likely to be of interest to researchers aiming to increase eradication rates, but the majority of data dwells on the role of probiotics. There is, however, some data suggesting a role for statins and pronase.

PROBIOTICS

Probiotics are considered safe. Thus, they are proposed as an adjuvant therapy to increase eradication rates and to decrease the side effects of therapeutic regimens, especially antibiotic-associated diarrhea. A meta-analysis published by Zhang *et al*[121] analyzed data from 45 RCTs and revealed that the addition of probiotics to a standard therapy was associated with an increased eradication rate (82.31% in probiotic group *vs* 72.08% in control group) and a lower incidence of adverse events. The mechanism of this action is probably related to the ability of probiotics to induce anti-inflammatory and anti-oxidative mechanisms that regulate the intestinal microbiota. Today, urease is considered a single possible target for probiotic action[122,123]. According to Ruggiero, it is more likely that probiotics exert indirect and non-specific, rather than direct and specific, anti-*H. pylori* activity[124].

STATINS

HMG-CoA reductase inhibitors have many pleiotropic effects. Therefore, Nseir *et al*[125] tested, in a small RCT, the hypothesis that the addition of simvastatin could improve eradication rates. According to this study, a better eradication rate (91% *vs* 72%) was observed in the group whose treatment included statin. Further studies are needed in this field.

PRONASE

Pronase is proteolytic enzyme that causes the degradation of gastric mucus, and its addition to standard eradication therapy was investigated in 1995 and 2002[126,127]. A single RCT by Gotoh used pronase with an eradication therapy (lansoprazole once daily, 500 mg of amoxicillin, 250 mg of metronidazole and 18000 tyrosine units of pronase thrice daily for 2 wk) and demonstrated an increased eradication rate in the group treated with pronase (ITT: 94% *vs* 76.5%, $P = 0.0041$)[126]. More validation is needed because the therapeutic regimens used in these studies are not currently standard eradication therapy protocols.

ADHERENCE TO TREATMENT

Patient adherence to treatment regimens is important for the successful eradication of *H. pylori*. According to the available data, successful eradication was observed in 96% of patients who took more than 60% of the prescribed medication[128]. Adherence to sequential therapy varies from 81%-98%, while adherence to concomitant therapy has been reported as 78.7%-100% in different studies[129-135]. The lowest adherence is observed in Spain[131], and the highest is observed in Taiwan[135], as seen in Table 1.

PASSIVE IMMUNIZATION

Passive immunization is effective in the prevention and treatment of various infectious diseases[136-138], making it a plausible strategy for the treatment of *H. pylori* as well. Data from animal studies has supported this concept, together with a previously reported protective effect from breastfeeding[139-141]. It is probable that specific antibodies inhibit the adherence of *H. pylori*. Clinical studies have reported conflicting data. Some studies have suggested that treatment with bovine antibodies could eradicate or decrease *H. pylori* colonization density[142,143], while others have failed to demonstrate this effect[144,145]. The first RCT to evaluate the efficacy and safety of specific anti-*H. pylori* polyclonal bovine IgA antibodies to reduce the intragastric bacterial load and gastritis activity in humans was performed by den Hoed[146]; the authors concluded that the antibody-based oral immunotherapy appears to be safe but ineffective because it did not significantly reduce *H. pylori* colonization density.

VACCINE

Active immunization against *H. pylori* infection would reduce the cost and potential complications of eradication therapy and is expected to lower gastric

cancer incidence. The development of a vaccine against *H. pylori* is complicated by the fact that the bacterium is noninvasive and remains strictly luminal without crossing the epithelium. An effective vaccine therefore must induce the appropriate Th memory cells that can be recruited to the mucosal surfaces. An effective vaccine against *H. pylori* should consist of appropriate bacterial antigens, an effective and safe adjuvant, and the route of delivery should be adequate. Different protocols have been tested using different antigens, adjuvants and application routes.

ANIMAL MODELS

In studies on animal models using classical immunization protocols, *H. pylori* lysates or *H. pylori* proteins were used, and plausible candidate antigens were identified (*i.e.*, urease, catalase, VacA, CagA, NapA, HpaA, AlpA and BabA). Better protection resulted from combinations of antigens[147,148]. Recently, new bacterial antigens have been proposed as candidates for vaccine development, including 20 kD outer membrane lipoprotein Lpp20[149], AhpC (alkyl hydroperoxide reductase)[150] and antioxidant proteins (*e.g.*, superoxide dismutase and catalase)[151].

Some studies that have used attenuated *Salmonella strains*, which express *H. pylori* ureA and ureB antigens, as delivery systems have demonstrated significant protection, both with intranasal and oral administration[152,153]. Recently designed *Salmonella* vector approaches use outer inflammatory protein A[154] for oral therapeutic immunization in addition to CagA, VacA and UreB in the vector[155]. This OipA-*Salmonella* based approach seems to be effective at both inducing OipA-specific antibodies and reducing *H. pylori* colonization. Overall, the *Salmonella-based* approach seems to be successful in animal models.

A polio virus-based vaccination using urease B had both prophylactic and therapeutic efficacy[156].

A multi-epitope approach is the basis of several new vaccine candidates and is described in detail in several studies. Li *et al*[157] used B and T cell epitopes that were generated by software prediction aimed at inducing both humoral and cellular immune response. Epivac uses proteins consisting of predicted T cell epitopes from HpaA-, UreB- and CagA[158]-inducing serum but unfortunately no mucosal immunity. The induction of mucosal immunity could be an effective *H. pylori* vaccination protocol. Promising results were obtained in a study using chimeric flagellin consisting of the hypervariable domain of *H. pylori* FlaA and the C- and N-terminal segments of *Escherichia coli* (*E. coli)* flagellin that was designed by Mori[159]. The chimeric flagellin was designed to maintain *H. pylori* specificity and gain TLR5 activity.

The vaccine in animal models was administered using different routes such as intranasal, oral, intramuscular, subcutaneous, rectal and intraperitoneal. Different adjuvants have been tested in animal models, *i.e.*, cholera toxin (CT) or heat-labile enterotoxin (LT), but the clinical use of these strong mucosal adjuvants is limited in humans because of their toxicity. A possible solution to this problem is to detoxify the adjuvant while maintaining its stimulatory effect[160]. However, these adjuvants have not been used in human clinical trials against *H. pylori*[161].

STUDIES IN HUMANS

In humans, majority of clinical studies have used recombinant urease as an antigen. In humans, clinical trials have tested the ability of experimental vaccines to eradicate existing *H. pylori* infection or to prevent the colonization of the gastric mucosa after introduction of the bacterium in an experimental challenge[162].

In *H. pylori*-infected asymptomatic individuals, oral immunization was well tolerated but did not lead to a specific immune response[163], while adding LT induced an immune response[164] and reduced *H. pylori* colonization[165]. Diarrhea occurred as a consequence of LT toxicity, but limiting the amount of LT was not effective, in that it resolved the side effects of LT but also reduced the immune response. Rectal administration of urease and LT induced a weak immune response[166].

Urease-expressing *Salmonella*-based delivery vectors did not prove to be effective in humans, which is in opposition to findings in animal models[167,168], because immune reactions were undetectable. Initial data on the use of the urease-expressing *Salmonella* vaccine strain Ty21a was disappointing because the immunologic response in *H. pylori*-negative volunteers was weak. T cell memory was observed in very few subjects, and no urease-specific antibodies were detected[168]. However, further investigation revealed that administration of multiple doses of a *Salmonella*-Ty21a based recombinant vaccine or the use of another recombinant strain that expressed the HP0231 *H. pylori* antigen resulted in the development of an immune response specific to *H. pylori* infection[169] and a decrease in the number of bacteria in gastric biopsies, both in vaccinated subjects and in the control group. Attenuated vaccines are well tolerated and might be the correct direction for further research.

There is evidence that efficacy can be improved *via* multivalent subunit vaccines[170]. A multivalent subunit vaccine consisting of CagA, VacA and NapA[171] was administered intramuscularly to *H. pylori*-negative volunteers, in whom it induced both humoral and cellular immune responses without side effects. Unfortunately, protection from *H. pylori* in a clinical setting did not differ between the placebo and the vaccine groups[172].

Recently, CagL protein was proposed as promising candidate for use in a subunit vaccine[173].

MUCOSAL ADJUVANT

The use of strong mucosal adjuvants that have been tested in animal models is limited in humans because of their toxicity (*i.e.*, CT and LT).

In the field of mucosal adjuvants, important results have been published in recent years. Nedrud *et al*[174] demonstrated that the intranasal administration of a mucosal adjuvant, CTA1-DD (a derivative of the cholera toxin), is safe, effective and protected against a live *H. pylori* challenge in mice. The use of heat shock proteins (Hsp) as the mucosal adjuvant in a *H. pylori* vaccine has also been reported[175]. This vaccine was administered *via* respiratory route and induced systemic and mucosal antibodies; protective immunity was generated with a milder post-immunization inflammation. Nevertheless, sterilizing immunity was not achieved[175]. Promising results were obtained when a nontoxic double mutant of an *E. coli* toxin (R192G/L211A) (dm2T) was used as the mucosal adjuvant[176]. Altman *et al*[177] suggested that carbohydrate-based vaccines against *H. pylori* should use dextran-based conjugates, and Zhang *et al*[178] suggested recombinant *Lactococcus lactis* as a vector because the bacterium is already used in dairy products.

ROUTE OF ADMINISTRATION

A report from Shirai *et al*[179] indicated that salivary antibody formation is a critical factor for successful vaccination against *H. pylori.* This was supported by the findings of Ng *et al*[180], who confirmed increased levels of salivary IgA without an increase in mucin production or cytokine level. Intranasal application in animal models seems to be promising and effective[174]. A multivalent subunit vaccine consisting of CagA, VacA and NapA[172] was administered intramuscularly. The sublingual[176] route appears to be promising, while an oral route of administration has repeatedly been tested with variable effects that depend on the antigen and the adjuvant or vector used[163-165,169,178]. Rectal administration was tested once and the results were somewhat disappointing[166].

OVERCOMING IMMUNITY ISSUES

To develop an effective vaccine for *H. pylori*, a better understanding of protective immune responses from data on animal models and a better understanding of host responses is needed. The identification of the underlying mechanisms that prevent a host from clearing the infection could help in the development of an effective vaccine.

The T helper cell response is an important part of the protective immune response because data on animal models suggest that mice lacking antibody molecules, including mucosal immunoglobulin IgA, are well-protected by vaccinations[181-183], while mice with deficient cellular immunity are not protected[182,184]. These data suggest that the T helper cell response is crucial for the induction of protective immunity, even in the absence of other forms of adaptive immunity[161]. This is based on the fact that Th1 or Th17 cell-induced increases in inflammation lead to the development of protective immunity. Therefore, a successful vaccine should induce a strong Th1 or Th17 response. According to Zawahir, possible multiple effector mechanisms that can eradicate *H. pylori* are not adequately defined. Therefore, vaccine efficacy could be improved through the enhancement of Th1 or Th17 responses to *H. pylori*[161]. It seems that the host immune response is age dependent, because a weaker Th1 response is observed in children[185].

In isolated cells, the removal of $CD25^+$ T cells before *H. pylori* stimulation increases the IFNγ response[186]. The results obtained in an animal model revealed that T regulatory cells suppress the active host immune response to *H. pylori* infection[186-190]. Because the majority of infected individuals do not have an *H. pylori*-associated disease, the host might not recognize the bacterium as dangerous and may suppress the immune response. Another possible target for improvement of vaccine efficacy is therefore overcoming the host predisposition to reducing immune responses to bacteria. A vaccine should increase the host cellular immune response, as supported by findings in animal models where bacterial load was reduced using IL-17[191] and IL-12[192].

Recent publications by Muhsen *et al*[193,194] demonstrated higher seroconversion rates to a typhoid vaccine in *H. pylori*-infected subjects, and the authors also demonstrated that gastric *H. pylori*-associated inflammation promoted seroconversion. Based on these findings, the concept that active *H. pylori* infection could be beneficial for the efficacy of other vaccines becomes attractive, and further research is needed in this area.

CONCLUSION

Timely eradication of *H. pylori* infection is, at present, the single available evidence-based strategy for reducing *H. pylori*-related gastric cancer risk, incidence, and subsequent morbidity and mortality. It is mandatory to eradicate the infection before irreversible mucosal damage occurs. Eradication should precede the development of severe atrophic changes.

Future possible strategies include the development of an effective vaccine. It is rather disappointing that although some of the experimentally tested vaccines have shown promising results, there is limited funding and financial support from the pharmaceutical industry. At the moment, another possible strategy is the development of new antimicrobial agents that would be effective for eradication therapy, but these are long-lasting and time-consuming studies. Therefore, at present, it is advisable to insist on patient compliance

during eradication therapy because this is the area where improvement is possible.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Mitogen-activated protein kinase signaling pathway and invasion and metastasis of gastric cancer

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Abstract

The mortality rate of gastric cancer worldwide is as high as 70%, despite the development of novel therapeutic strategies. One reason for the high mortality is the rapid and uninhibited spread of the disease, such that the majority of patients are diagnosed at a stage when efficient therapeutic treatment is not available. Therefore, in-depth research is needed to investigate the mechanism of gastric cancer metastasis and invasion to improve outcomes and provide biomarkers for early diagnosis. The mitogen-activated protein kinase (MAPK) signaling pathway is widely expressed in multicellular organisms, with critical roles in multiple biological processes, such as cell proliferation, death, differentiation, migration, and invasion. The MAPK pathway typically responds to extracellular stimulation. However, the MAPK pathway is often involved in the occurrence and progression of cancer when abnormally regulated. Many studies have researched the relationship between the MAPK signaling pathway and cancer metastasis and invasion, but little is known about the important roles that the MAPK signaling pathway plays in gastric cancer. Based on an analysis of published data, this review aims to summarize the important role that the MAP kinases play in the invasion and metastasis of gastric cancer and attempts to provide potential directions for further research and clinical treatment.

Key words: Mitogen-activated protein kinase; Gastric cancer; Signaling; Invasion; Metastasis

Core tip: The mortality rate of gastric cancer is as high as 70% worldwide due to the rapid and uninhibited metastasis and invasiveness of the disease. Although the relationship between the mitogen-activated protein kinase (MAPK) signaling pathway and cancer metastasis and invasion has been widely researched, few studies have focused on gastric cancer. Here we

review the function of the three central kinases of the MAPK signaling pathway, ERK, JNK and p38, in the metastasis and invasion of gastric cancer, and we attempt to provide support for further in-depth study and clinical application.

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INTRODUCTION

The mitogen-activated protein kinase (MAPK) is a type of serine/threonine protein kinase that is able to respond to multiple extracellular stimuli. Growth factors, insulin, environmental factors, and cytokines may all activate the MAPK kinases and lead to a broad intracellular response through the MAPK signaling pathway. The MAPK signaling pathway is one of the earliest signaling pathways to emerge during evolution and has been evolutionarily conserved. The pathway comprises the MAPK cascade protein kinases. Each typical single MAPK cascade pathway includes at least three core kinases, MAP3K, MAPKK, and MAPK[1]. The MAPK pathway exists in almost all eukaryotes and is involved in many cellular activities, including the regulation of gene expression, mitosis, metabolism, cell proliferation, apoptosis and cellular movements[2]. In view of the critical role of the MAPK pathway in cellular activities, the dysregulation of MAPKs often directly or indirectly leads to disease.

Local invasion and metastasis cause the majority of cancer deaths, and the distant metastasis of cancer accounts for the death of over 90% of patients[3]. Gastric cancer spreads easily to the adjacent organs and tissues, including the liver, pancreas, colon, lungs and bone, *via* the lymphatic system[4]. In fact, although much clinical effort is made, gastric cancer still has a mortality rate as high as 70%, because most gastric cancer patients are in the metastasis stage at the time of diagnosis[5].

Detachment of cancer cells from the primary tumor is the first step in tumor invasion and metastasis; subsequently, detached tumor cells are transported into and invade the blood and lymphatic vessels; and finally, cancer cells escape from the lumina of these vessels, settle in the target organs, and grow into macroscopic tumors[6-8].

The molecular process of tumor invasion and metastasis involves several essential events, such as the degradation of the extracellular matrix and the adhesion of cancer cells to the target with the help of focal adhesion kinase (FAK) and matrix metalloproteinases (MMPs)[9,10]. Mitogen-activated protein kinases are involved in cell migration and invasion events partially by regulating the expression and activation of MMPs and FAK[11-13]. Moreover, the MAPK pathway participates in invasion and metastasis through other types of signaling pathways. The aim of this article is to provide an introduction to the role that the MAPK pathway plays in gastric cancer metastasis and invasion based on the published data and provide recommendations for future research.

ERK1/2 AND GASTRIC CANCER INVASION AND METASTASIS

Introduction to ERK and the ERK/MAPK pathway

ERK is one of the first mammalian MAPK genes to be identified and cloned. The cDNAs of ERK1 and ERK2 were both cloned as early as the 1990s, and they share up to 83% of identical amino acids[14,15]. Moreover, there are other isoforms of ERK, including ERK3, ERK4, ERK5, and ERK7/8[16]. In this section, we will mainly discuss the most important members that play critical roles in cancer invasion and metastasis, ERK1/2.

The integral ERK/MAPK pathway can be roughly divided into three levels, which are summarized in Figure 1. Raf isoforms are the most well-studied kinases, constituting the highest level of the ERK/MAPK pathway, and are also known as MAPKKKs. Extracellular growth factors, insulin and G-proteins may activate the MAPKKKs by directly binding to the N-terminus of the Raf protein and transforming its structure through phosphorylation. Then, the activating signal is passed to the MAPKKs through the phosphorylation of two serine residues on the MEK1 or MEK2 protein. The signal is finally transmitted to ERK by MEK1/2 through the phosphorylation of tyrosine and threonine residues[17]. When the entire signaling pathway is completely activated in order, hundreds of ERK/MAPK pathway substrates are phosphorylated, and these events affect ERK-dependent cellular activities, including cell proliferation, differentiation, neuronal flexibility, cell viability, cellular stress response and apoptosis[2].

ERK functions in gastric cancer

The dysregulation of ERK/MAPK occurs in various human diseases, including neurodegenerative diseases, developmental disorders, metabolic diseases, and cancer[18-22]. In the last decade, scientists increasingly focused on the relationship between the ERK/MAPK pathway and tumor genesis and progression, because it was found that mutation or abnormal activation of the ERK/MAPK pathway exists in over half of human cancer types[23]. As an upstream binding kinase of the ERK/MAPK pathway, Ras was reported to mutate to an oncogenic form in more than 15% of human cancers. Additionally, B-RAF mutated in 66% of malignant

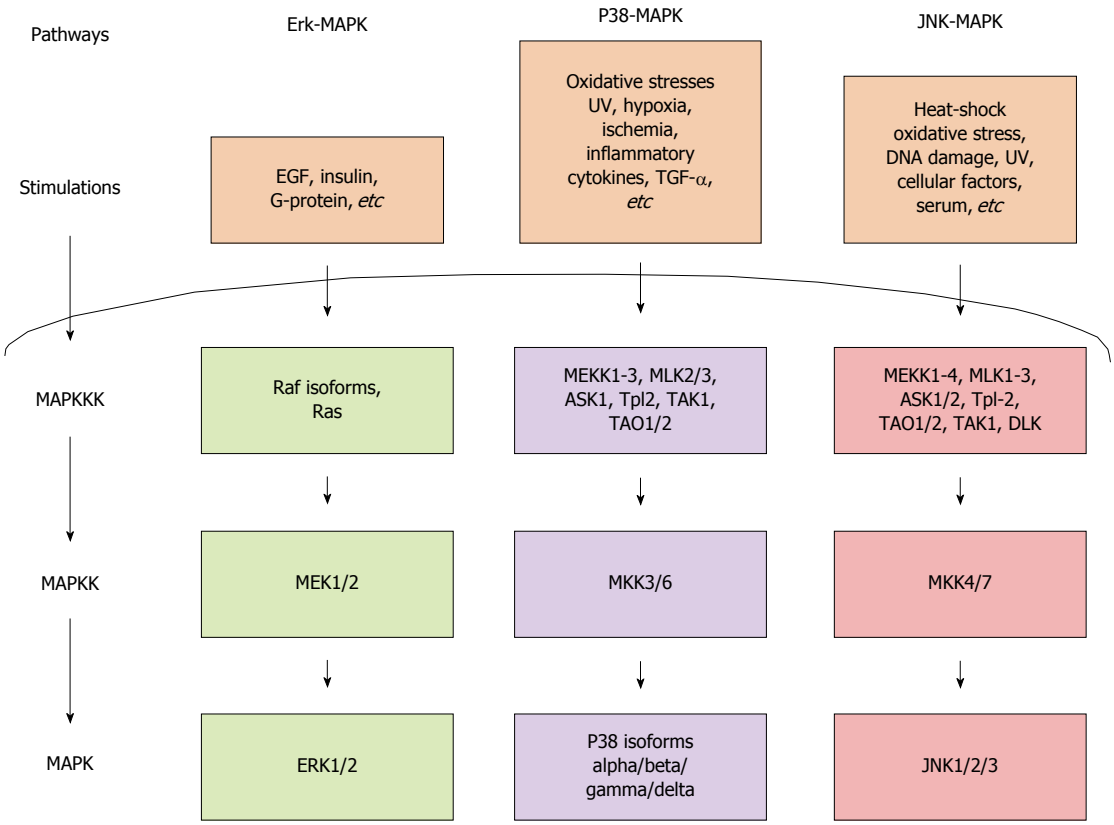

Figure 1 The integral extracellular signal-regulated kinase/mitogen-activated protein kinase pathway. ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase.

melanomas. Point mutations of the Ras and B-RAF genes cause dysregulation of the ERK/MAPK pathway and abnormal cellular motility, which primarily lead to the migration and invasion of cancer cells[24].

Many studies have elucidated that the ERK/MAPK pathway plays an active role in the invasion and metastasis progression of some malignant tumors. The pathologic process of tumor invasion and metastasis requires cell motility. The alteration of cellular adhesiveness directly affects cell movement. The epidermal growth factor receptor (EGFR)-induced disassembly of focal adhesions is regulated by activating the ERK/MAPK pathways[25]. Another study of epithelial cells demonstrated that ERK *in vitro* was closely correlated with metastasis both in the TGF-beta and the RAS/MAPK pathways[26]. Metastasis induced by dysregulation of ERK was also demonstrated in animal models. All of the three domain mutations V12S35, V12G37, and V12C40 of Ras are able to induce tumor genesis, but only the V12S35 mutation, which affects the activation of the ERK/MAPK pathway, rather than the other two domain mutations of Ras, induced tumor metastasis in mouse models[27]. This study showed that Ras could induce tumor genesis independently of the ERK/MAPK pathway; however, the ERK/MAPK pathway is indispensable in Ras domain mutation-induced tumor metastasis.

Recently, an increasing number of studies have revealed that the ERK/MAPK pathway is involved in regulating cellular mobility in gastric malignant tumors and gastric cancer cell lines. ERK may mediate the activity of MMPs, which in turn influences gastric cancer cell migration and invasion[28-30]; conversely, many factors upstream of the ERK/MAPK pathways, such as interleukin-22 (IL-22), RASAL1, phophatase of regenerating liver 3 (PRL3), NAIF1, CCDC134, and ZIC1, may influence invasion and migration in gastric cancer cell lines through the ERK/MAPK pathway[30-35]. Currently, most studies focus on the role of the ERK/MAPK pathway in gastric cancer cell lines. Evidence in tissues and animal models is sparse, and further research is needed.

STRESS ACTIVATED MAPK

P38

Introduction of p38: p38 alpha, beta, gamma and delta are the four well-known members of the p38 MAPK family, of which p38 alpha and p38 beta are expressed ubiquitously, whereas the p38 gamma and p38 delta have more restricted expression patterns[16].

The mammalian p38 MAPK pathway is affected by various environmental stressors, including oxidative stress, UV, hypoxia, ischemia, as well as inflammatory cytokines and transforming growth factor-α (TGF-α)[36]. MEKK1-3 (MEK kinase 1-3), MLK2/3 (mixed lineage

kinase 3), ASK1 (apoptosis signal regulating kinase 1), Tpl2 (tumor progression loci 2), TAK1 and TAO1/2 (thousand and-one amino acid) are all MAPKKKs in the P38 MAPK pathway[37]. These MAPKKKs activate p38 by phosphorylating it at the Thr-Gly-Tyr motif through selective activation of MKK3/6. MKK6 phosphorylates all four members of the p38/MAPK family, whereas MKK3 phosphorylates p38 alpha, p38 gamma, and p38 delta but not p38 beta[37]. p38 is found in both the nucleus and the cytoplasm and translocates into the nucleus in response to certain types of stress. The P38 kinase affects certain types of downstream substrates after being activated, including cPLA2, MNK1/2, MK2/3, HuR, Bax, and Tau in the cytoplasm and ATF1/2/6, MEF2, Elk-1, GADD153, Ets1, P53 and MSK1/2 in the nucleus[37].

P38 and gastric cancer invasion and metastasis

The p38 pathway has been implicated in a range of complex biological processes, such as cell proliferation, differentiation, migration, and apoptosis. Dysregulation of P38 in patients with solid tumors, such as prostate cancer, breast cancer, bladder cancer, liver cancer and lung cancer, is associated with advanced stages and low survival rates[38]. The p38 signaling pathway exhibits some anti-tumor effects in xenograft experiments[39,40]. In hepatocellular carcinoma, the activity of P38 is down-regulated in the cancer tissue compared with the adjacent normal tissue, and the tumor volumes are related to the p38 activity[41]. As a result, tumor cells must modulate p38 activity to achieve metastasis and invasion.

The epithelial-mesenchymal transition (EMT) process plays an important role during the initiation of metastasis. P38 signaling is involved in the regulation of EMT in several ways. For example, p38 participates in regulating the EMT activity in mammary epithelial cells and in primary ovarian tumors by separately regulating the phosphorylation of Twist1 and the expression of Snail[42,43]. P38 is also involved in ROS-triggered EMT, and this process may be reversed by the introduction of the p38 inhibitor SB203580[44,45]. The MMP protein family plays a critical role in remodeling the extracellular matrix during metastasis. There are many studies of the relationship between p38 and the expression of MMP family members in liver, prostate, breast and skin cancer cell lines. Following inhibition of p38 in these cell lines, cellular invasion decreases[46-48]. Many small molecules can regulate the metastasis and invasion of gastric cancer through regulation of the p38 signaling pathway. For example, S100A8 and S100A9, the low-molecular-weight members of the S100 family of calcium-binding proteins, induced gastric cancer cell migration and invasion involving p38 and NF-κB, whereas they did not affect cell proliferation and cell viability, which leads to an increase in MMP2 and MMP12 expression[49]. In addition, the widely used anti-tumor drug baicalein also inhibits gastric cancer cell invasion and metastasis by reducing cell motility and migration *via* suppression of the p38 signaling pathway[50].

C-JUN N-TERMINAL KINASE

Introduction to c-Jun N-terminal kinase

The c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), was first identified and named as c-Jun transcription factor. The three isoforms of JNK, JNK1/2/3, were cloned in the mid-1990s. These three isoforms are encoded by three different genes, sharing more than 85% homology; these isoforms result from more than 10 splices and have molecular weights varying from 46 kD to 55 kD[51,52]. JNK1/2 is widely expressed in mammary tissues, whereas JNK3 is expressed mainly in nervous tissues, testis, and cardiac muscle[53]. All members of the JNK family are activated by various stimulating factors, such as heat shock, oxidative stress, DNA damage, UV, cellular factors, and serum[54]. The MAPKKKs in the MAPK/JNK signaling pathway include MEKK1-4, MLK1-3, Tpl-2, DLK, TAO1/2, TAK1, and ASK1/2. These MAPKKKs activate the MAPKKs, such as MKK4 and MKK7, through phosphorylation. Subsequently, activated MKK4/7 will activate JNK by phosphorylating its threonine and lysine residues[16].

JNK and gastric cancer metastasis and invasion

JNK serves as an important kinase that regulates cellular activity; current research suggests that JNK plays opposite roles in cancer initiation and development[55]. Experiments demonstrated that a mouse model with a JNK2 deficiency shows a lower incidence of tumor initiation[56]; however, a mouse model with a JNK1 deficiency may generate tumors more frequently compared with control mice[57]. Therefore, small molecular inhibitors of JNK could not be easily used in cancer therapy.

Additionally, JNK is also involved in gastric cancer metastasis and invasion. Recombination in Erdr1 suppresses the ability of the gastric cancer cell line SNU-216 to metastasize by up-regulating E-cadherin through JNK pathway activation, and this event is reversed by treating the cells with the JNK inhibitor SP600125[58]. JNK is also involved in TGF-beta1-induced invasion and metastasis of gastric cancer cells. As an important mediator of the tumor response to TGF-beta, fascin1 functions through the TGF-beta1-JNK/MAPK pathway to regulate gastric cancer invasion and metastasis[59].

CONCLUSION

The in-depth mechanisms and molecular signaling pathways in gastric cancer invasion and metastasis are complex. As the vital signaling pathway in regulating cellular vitality, the MAPK pathway plays im-

portant roles in cancer invasion and metastasis. Over the past decades, studies increasingly revealed that the MAPK pathway is involved in regulating gastric cancer invasion and metastasis; however, systematic research, especially in animal models, is still needed. Further studies in this field could provide a better understanding of gastric cancer invasion and metastasis, as well as uncover novel targets and effective clinical treatments.

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Competing endogenous RNA networks and gastric cancer

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Abstract

Recent studies have showed that RNAs regulate each other with microRNA (miRNA) response elements (MREs) and this mechanism is known as "competing endogenous RNA (ceRNA)" hypothesis. Long non-coding RNAs (lncRNAs) are supposed to play important roles in cancer. Compelling evidence suggests that lncRNAs can interact with miRNAs and regulate the expression of miRNAs as ceRNAs. Several lncRNAs such as H19, HOTAIR and MEG3 have been found to be associated with miRNAs in gastric cancer (GC), generating regulatory crosstalk across the transcriptome. These MRE sharing elements implicated in the ceRNA networks (ceRNETs) are able to regulate mRNA expression. The ceRNA regulatory networks including mRNAs, miRNAs, lncRNAs and circular RNAs may play critical roles in tumorigenesis, and the perturbations of ceRNETs may contribute to the pathogenesis of GC.

Key words: Competing endogenous RNA; Competitive endogenous RNAs networks; Gastric cancer; MicroRNA response elements; Perturbation

Core tip: Competitive endogenous RNAs (ceRNAs) share microRNA (miRNA) response elements and compete common miRNAs, thereby regulating each other's expression. The ceRNA regulatory networks including mRNAs, miRNAs, long non-coding RNAs and circular RNAs play critical roles in tumorigenesis, and the perturbations of ceRNA networks may contribute to the pathogenesis of gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide and is a major cause of cancer-related mortality in China[1]. Since the carcinogenesis in GC is a complex process with etiological factors, genetic and epigenetic alterations involved[2], the molecular basis of GC, especially efforts to identify clusters of predictive markers, has been widely studied. Previous studies have demonstrated that several genetic abnormalities such as aberrant genes, copy number variants (CNV), microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) were involved in the initiation and progression of GC[3], but the pathogenic mechanisms contributing to biological feature of GC remain to be elucidated.

Non-coding RNAs (ncRNAs) refer to a class of RNAs with no protein-coding function that are widely expressed in organisms, including small ncRNAs such as miRNAs and lncRNAs, both of which play important roles in the post-transcriptional regulation[4]. In fact, miRNAs have been extensively studied in the field of oncology, and emerging evidence suggests that miRNA-mediated regulation plays crucial roles in tumor cell biological processes, such as cell proliferation, migration and invasion[5]. Furthermore, aberrantly expressed miRNAs have been discovered in diverse diseases including GC.

The competing endogenous RNA (ceRNA) hypothesis postulates that RNAs that share miRNA response elements (MREs) in their 3'-untranslated regions (UTRs) can influence the expression of miRNAs, inducing gene silencing[6]. While recent several studies have demonstrated that lncRNAs can harbor MREs and interact with other RNA transcripts such as ceRNAs[7,8]. The complex crosstalks of ceRNAs have been found in many different cancer types including GC. Above all, functional interactions and disequilibrium of ceRNA networks (ceRNETs) may contribute to disease pathogenesis[9]. This review discusses the features of ceRNETs and the functional roles and regulatory interactions of ceRNETs in the development of GC.

FEATURES OF CERNETS

In view of ceRNA hypothesis, three studies were reported in 2011 from Columbia University, Harvard Medical School and the University of Roma La Sapienza, which verified the ceRNA hypothesis from many aspects and further confirmed the establishment of regulatory mechanism based on ceRNAs[10]. The discovery of ceRNA mechanism provocatively subverts traditional meaning of the mRNA function, which means that mRNAs not only have the function of encoding proteins, but also participate in the gene regulation processes[11,12]. Transcriptional regulatory networks based on ceRNAs, not only enrich the biological pathway in the existing networks, but also expand the function of the human genome. Regulation members of ceRNETs consist of mRNAs, miRNAs, lncRNAs and circular RNAs (circRNAs). Notably, miRNAs and MREs are considered two important elements in the ceRNA hypothesis. The former is core motivation, while the latter is structural foundation.

Protein coding genes

So far, the number of protein coding genes in human genome has been found to be approximately 20000[13]. And most of mRNAs are covered in MREs[14,15]. Recent studies have demonstrated that many mRNAs are validated as ceRNAs, so mRNAs play an essential role in ceRNETs.

The function of miRNAs can be influenced by their target mRNAs for limited MREs within each cell. For a given mRNA, its upregulation can lead to the increasing number of MREs, which exceeds their targeting miRNAs. So each mRNA can act as an inhibitor for shared miRNAs. To date, *PTEN* that competes with various ceRNAs has been widely validated in a variety of advanced and metastatic cancers[16]. This tumor suppressor gene is involved in the regulation of cell proliferation, migration and apoptosis. The occurrence of *PTEN* inactivation was closely related to GC stage[17]. Recently, a study has successfully validated that a protein-coding transcript ZEB2 plays a role as a PTEN ceRNA in melanoma, which suggests that ZEB2 is involved in regulation of PTEN expression in an miRNA-dependent manner[18]. In another study, Tay *et al*[19] have validated that endogenous protein-coding transcripts VAPA and CNOT6L possessing tumor-suppressive properties could regulate *PTEN* through the disturbance of the PI3K/AKT signaling pathway.

Studies utilizing high-throughput technologies such as microarray and NGS for gene expression profiling have increased the discovery of predictive and treatment biomarkers. So far, numerous driver genes have been involved in gastric tumorigenesis. *P53* mutations, which were observed in a large proportion of tumors, had a crucial and early role in gastric carcinogenesis of intestinal type[20,21]. E-cadherin gene (*CDH1*) inactivating mutations were identified in diffuse GC and carriers with *CDH1* mutation(s) were more likely to have an increased risk of developing GC[22,23]. Furthermore, previous studies found that frequent *ARID1A* alterations were detected by exome sequencing in two specific molecular subtypes of GC[24,25]. In addition to the previously known mutations, a recent study of whole-genome sequencing (WGS)[26] has identified new driver genes of gastric adeno-

carcinoma. *MUC6*, which encodes gastric mucin, was significantly mutated. And *RHOA* mutations were observed in diffuse-type GC. These emerging drivers, together with other genes including *CTNNA2, GLI3,* and *RNF43*, are potential players in the perturbed pathways of GC. Although dozens of genes have been found, their roles in tumorigenesis in GC remain to be elucidated. Concurrently, these driver genes could be ceRNAs, which act as mediators, involved in the regulation of ceRNETs.

MiRNAs

MiRNAs are small noncoding RNAs that regulate the expression of various genes by inhibiting or degrading target mRNAs[27]. It is estimated that 30% of genes in the human genome were regulated by miRNAs[28,29]. MiRNAs containing MREs are shared by all ceRNAs. Accumulating evidence supports that a new layer of regulation of ceRNETs produces a tendency to be mediated by miRNAs. Multiple miRNAs can regulate different MREs in mRNA transcripts, and each miRNA can inhibit hundreds of transcripts, so miRNAs act as mediators in huge transcriptional and signaling networks[30]. This regulatory mechanism constitutes the basis of ceRNA interplay networks.

Emerging evidence suggests that aberrant miRNAs participate in the pathogenesis of GC, mainly by regulating the expression of oncogenes and tumor suppressors. Overexpression of miR-21, a known oncogenic miRNA, could enhance cell proliferation and inhibit apoptosis in cancer patients[31,32]. The target genes of miR-21 such as TMP1, PTEN and RECK were confirmed in several studies by different technological methods[33,34]. These findings support that miR-21 that function *via* the regulation of target genes mediates oncogenic processes in GC. Dysregulated miRNAs (miR-125a, miR- 199a, and miR-100) were considered to be important factors in the regulation of GC[35-37], suggesting that they may play different functions in different sites.

Gastric carcinogenesis is a multi-stage process, in which molecular expression and signaling pathway disturbances are involved[38]. Chronic inflammation is a driving factor promoting the malignant transformation. Specifically, *Helicobacter pylori* (HP)-induced gastritis is a risk factor for GC. The expression of certain miRNAs including miR-21, miR-155, miR-194, miR196, miR-218, and miR-223 has been found to be increased in GC with HP infection. Saito *et al*[39] noted that the overexpressed miR-155 acting as an important negative regulator modulates the inflammatory responses in GC induced by HP infection. Additionally, Wang *et al*[40] reported that a great dependence was confirmed between miR-106a and lymph node metastasis in GC. Another study also[41] discovered that HP infection could lead to decreased expression of Let-7, which increases the expression of oncogene *Ras*. As stated above, aberrant miRNAs play central roles in ceRNETs by regulating target genes.

LncRNAs

LncRNAs play regulatory roles and are dysregulated in a variety of tumors. However, the potential mechanisms of how lncRNAs alter in GC remain largely undefined. An increasing number of lncRNA transcripts emerged recently as ceRNAs have been implicated in GC.

In the research of GC, some lncRNAs are upregulated and act as oncogenic genes, including H19 and HOTAIR, while others are downregulated and function as suppressor genes, such as growth arrest-specific transcript 5 (GAS5) and maternally expressed gene 3 (MEG3). H19, a typical onco-lncRNA, was dysregulated in many cancers[42-44]. Park *et al*[45] reported that upregulated H19 can promote the development of GC by regulating the activity of *P53*. Recently, several studies[46] have demonstrated that HOTAIR may participate in the progression and metastasis of GC, and can be used as a therapeutic target for GC. GAS5, another famous lncRNA, plays a tumor-suppressive role in tumor formation. Significant downregulation of GAS5 could promote tumor cell proliferation by regulating expression of p21 and E2F1 proteins[47]. In addition, MEG3 was frequently studied in GC. Decreased expression of MEG3 could regulate cell proliferation and differentiation by interacting with *p53, Rb,* and *VEGF*[48]. Additionally, MEG3 may be associated with poor prognosis of GC by increasing the spread of cancer cells[49].

The key step in cancer research is to discover lncRNAs associated with specific diseases. At present, the screening of lncRNAs *via* chip analysis is a quick and accurate method. Song *et al*[50] demonstrated that 135 lncRNAs were dysregulated in GC tissues by microarray analysis, and H19 and uc001lsz were markedly expressed. The use of qRT-PCR also confirmed that the overexpression of H19 was closely related to GC, and uc001lsz might be a potential diagnostic marker for early GC. By means of expression profile analysis, Cao *et al*[51] identified 88 abnormally expressed lncRNAs including LINC00152, SNHG3, GAS5 and LINC00261. Additionally, Park *et al*[52] detected 31 differentially expressed lncRNAs using transcriptomics data, which further suggested that down-regulated BM742401 was closely related to poor survival of GC, and could be used as a therapeutic target to improve the prognosis of GC.

CircRNAs

CircRNAs are a special kind of endogenous RNAs featuring stable structure and high tissue-specific expression[53]. Instead of nonlinear RNAs, circRNAs are more common features[54]. So far, thousands of circRNAs have been found in human cells. The newly discovered circular RNAs can act as ceRNAs that affect the regulation of gene expression.

At present, studies on circRNAs are relatively few. CircRNAs functioning as miRNA sponges may play an important role at the level of miRNA fine tuning[55]. Hansen *et al*[56] suggested that CDR1 (cerebellar degeneration-related protein 1), known as ciRS-72011, was perceived as a ceRNA. Unlike other transcripts, CDR1 containing more than 70 MREs plays a role in regulation by interacting with miRNAs. By functional approaches, CDR1 was found to be overexpressed as a ceRNA that bound miRNAs, thus inhibiting the activity of miR-7[57]. Additionally, the study also discovered that 16 MREs were shared between miR-138 and a circRNA transcript derived from the testis determining gene (sex-determining region Y, Sry), which could have an miRNA sponge effect in regulating gene expression by inhibiting the activity of miR-138. In general, circRNAs are difficult to be degraded by enzyme for the feature of stable configuration and high abundance, which brings the regulatory function of cirRNA into full play.

Currently, circRNAs have been involved in several types of diseases[58,59] including GC. A study discovered that a typical circRNA, hsa_ circ_002059, is significantly downregulated and may be a potential diagnostic marker in GC[60]. Given the fact that the interactions between circRNA and miRNA may be very common, with the recognition of more molecules, circRNAs research is likely to have a giant leap, which will make contributions to tumor biology.

PREDICTION OF CERNETS

The availability of transcriptome data of diverse cancers, together with bioinformatic tools and computational approaches, enables the prediction of ceRNETs. At present, research on ceRNETs is certainly in its infancy, but still has made some progress.

By a novel multivariate analysis method, a huge miR-mediated ceRNET including 248000 crosstalks was first observed in glioblastoma[61]. Based on a special algorithm, a recent study has constructed a breast cancer-specific ceRNET using the expression profiles of miRNAs and mRNAs[62]. Similarly, a computational approach[63] was explored to predict miRNA- mediated sponge interactions (MMI-networks) based on both normal and breast cancer expression data, separately. This study also found that ceRNETs may be significantly altered between normal and pathological breast tissues and lncRNA PVT1 was a key factor in the tumorigenesis of breast cancer. Interestingly, based on lncRNA microarray data of GC, Xia *et al*[64] first constructed a ceRNA regulatory network including 8 lncRNAs and 9 miRNAs using bioinformatics methods and confirmed this network using the data from six types of other cancers. Additionally, Basia *et al*[65] proposed to analyze the equilibrium and non-equilibrium properties of ceRNETs based on a stochastic model, while emphasizing the robustness and response-time to external perturbations of the network.

CeRNA database

At present, the most effective way to reveal ceRNAs' function is constructing ceRNETs. As increasing attention has focused on ceRNA research, ceRNA databases are constantly established. Sarver *et al*[66] developed a putative human ceRNA database ceRDB, which aimed to predict specific miRNA target genes related to ceRNAs. In ceRDB, the competing mRNAs were sorted by an interaction score based on the number of shared MREs among ceRNAs. The higher the score was, the more likely to be affected by ceRNAs the target mRNAs were. However, unlike the ceRDB database, which excluded information about lncRNAs. lnCeDB[67] provided a database of human lncRNAs that could potentially act as ceRNAs by computing a ceRNA score, which was a novel algorithm. Noteworthily, lncRNA-mRNA pairs with common shared miRNAs were available in this database. Additionally, based on ceRNA hypothesis, a web resource Linc2GO database[68] was established to provide comprehensive function annotations for human lincRNAs. starBase v2.0[69] stored the information about regulatory networks based on broadest experimental support, and this database provided potential interactions between miRNAs, mRNAs and lncRNAs. A newly developed database miRcode[70] was described to collect putative microRNA target sites based on complete GENCODE gene annotations and was used to predict the targets of miRNAs, including mRNAs and lncRNAs. The latest version of this database contained 10419 lncRNA genes. DIANA-LncBase database[71] attempted to depict putative miRNA-lncRNA interactions, providing annotations of miRNA targets on lncRNAs. Furthermore, ChIPBase[72] database platform aimed to unravel transcriptional regulatory relationships between lncRNAs/lincRNAs and miRNAs through the integration of ChIP-Seq data. In short, the effective use of these databases will help us seek for biomarkers, avoiding the blindness in practice (Table 1).

Conditions that ceRNETs work

It is well-known that ceRNETs play a role in cell culture. Recently, some conditions required for ceRNETs have been found. First, the concentration of the ceRNAs should be strongly emphasized. Expression changes of ceRNAs should be large enough to effectively eliminate or weaken the inhibition of miRNAs to ceRNAs. Second, the effectiveness of ceRNETs always depends on the number of shared miRNAs. It can be speculated that, in a network, the ceRNA having more binding preference to the shared miRNA will have more profound ceRNA effect on the components with less binding preference. In addition, taking tissue specificity into account, ceRNETs would also rest on density and subcellular distribution of RNAs in the cell[73]. The balance between shared miRNAs and targeted ceRNAs is critical for ceRNA activity and disruption of this balance can trigger internal crosstalks in ceRNETs. In general, alterations of one ceRNA may lead to joint

Table 1 Competitive endogenous RNA related databases

Database	Website	Ref.
ceRDB	http://www.oncomir.umn.edu/cefinder/	[66]
lnCeDB	http://gyanxet-beta.com/lncedb/	[67]
Linc2GO	http://www.bioinfo.tsinghua.edu.cn/~liuke/Linc2GO/index.html	[68]
starBase v2.0	http://starbase.sysu.edu.cn/	[69]
miRcode	http://www.mircode.org/	[70]
DIANA-LncBase	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=lncBase/index	[71]
ChIPBase	http://deepbase.sysu.edu.cn/chipbase/	[72]

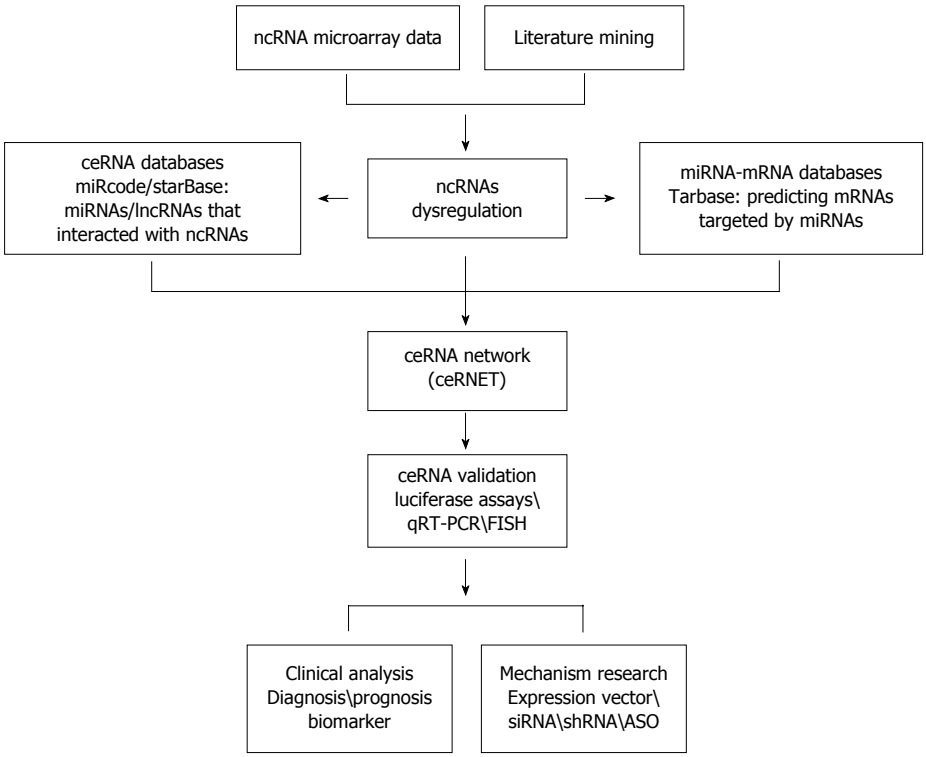

Figure 1 Flow chart for studying competing endogenous RNA network in cancers. ncRNA: Non-coding RNA; ceRNA: Competing endogenous RNA; shRNA: Short hairpin RNA.

consequences in huge ceRNETs and thus promote cancer.

Research methods of ceRNETs

Although ceRNA research is in its infancy, the current progress has gained much attention. The availability of RNA-seq data, along with bioinformatics tools, enables the prediction of ceRNETs. As show in Figure 1, we display a way to research ceRNETs.

First, multiple strategies can be applied to obtain differentially expressed ncRNAs in cancers, including literature mining and microarray analysis. Then by means of computational algorithm and public databases, we can predict potential connections in ceRNETs. Some miRNA target prediction databases such as Tarbase, TargetScan and miRecords can provide experimentally verified miRNA-gene interactions, which are stable foundation for ceRNETs. As a supplement, the CLIP-Seq datasets come in handy. These ceRNA databases encompass information about miRNA, mRNA, lncRNA, circRNA and pseudogene associations. Taken together, ceRNETs including lncRNAs, miRNAs, and mRNAs are constructed by invoking bioinformatics analysis.

Second, the precondition to study ceRNETs should be expression correlations, regulatory relationships, and shared MREs of ceRNA pairs. The validation of ceRNETs is considered to be an experimental framework for the biochemical method of ceRNA interactions. Based on the ceRNETs, the differentially expressed ceRNAs could be confirmed by qRT-PCR or fluorescence *in situ* hybridization (FISH).

Finally, functional studies should be conducted to investigate the dysregulation of ceRNAs in carcinogenesis. In brief, the effect of overexpression/interference expression among ceRNAs was assessed by function gain/deficit experiments such as siRNAs, shRNAs, and antisense oligonucleotides (ASO). Furthermore, these experimentations for validating the perturbation of ceRNAs should be investigated in

mouse models to get confirmed correlations.

CROSSTALKS BETWEEN CERNAS IN GC

In recent years, the mechanism of ncRNAs in tumors has become a hot research topic. At the same time, increasing evidence has indicated that ncRNAs can regulate each other and affect their function by binding to MREs of shared miRNAs[74]. Like the role of ceRNAs in GC, the disturbance of interactions between ceRNAs also plays a part.

Due to the ceRNA theory, the competition between lncRNAs and miRNAs makes indirect regulation possible. In light of the role in regulating target genes, miRNAs can exercise the similar function to negatively regulate the expression of lncRNAs, and thus exert a series of biological effects in GC. Yan *et al*[75] reported that MEG3 expression level was markedly reduced in both tissues and cell lines of GC, and further experiments found that transfection of MEG3 siRNA into cells could diminish the suppression of proliferation induced by overexpression of miR-148a, which suggested that miR-148a might decrease the expression of MEG3 by modulation of DNMT-1. Furthermore, another study[76] found that upregulated H19 could promote the proliferation of GC cells by binding miR-675, which inversely inhibited the tumor suppressor gene RUNX1. The interaction between H19/miR-675 and RUNX1 may serve as novel targets in the tumorigenesis of GC.

In addition to indirect regulation of ceRNAs, lncRNAs can have a direct interaction by invoking the "endogenous miRNA sponge" (miRNA sponge) to inhibit the activity of mRNAs, thus affecting the occurrence and development of tumors. Xu *et al*[77] discovered that upregulated lncRNA AC130710 played a crucial role during GC progression by targeting miR-129-5p. Liu *et al*[78] reported that the expression levels of upregulated HOTAIR and HER2 had a positive correlation in GC. And subsequent luciferase and RIP assays confirmed that HOTAIR served as an endogenous "sponge" to regulate the expression of HER2 by sinking miR-331-3p. These results indicate that possible crosstalks in ceRNETs may provide new clues for the mechanism of GC.

CONCLUSION

Recently, increasing evidence suggests that the dysregulation of ceRNA interactions including miRNAs and lncRNAs has been involved in disease etiology, including gastric cancer. In this review, we present and discuss the features of ceRNETs and crosstalks in GC, as well as the methods in the study of ceRNETs.

CeRNAs that function as key regulators have been implicated in many biological processes and the perturbation of ceRNETs may contribute to carcinogenesis. Given the complexity of ceRNETs, future works should focus on identifying the hubs that have significant influence on network balance or tumorigenesis. Despite some improvements in research field, the mechanisms of ceRNA crosstalks are still not fully elucidated. And there are still several considerations limiting the applications of ceRNETs. With the development of computational methods, research techniques and abundance of all components in ceRNETs, we anticipate that ceRNETs will provide a new avenue for the research of GC, and shed light on complex mechanisms underlying malignant processes.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Colitis-associated colon cancer: Is it in your genes?

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Abstract

Colitis-associated colorectal cancer (CA-CRC) is the cause of death in 10%-15% of inflammatory bowel disease (IBD) patients. CA-CRC results from the accumulation of mutations in intestinal epithelial cells and progresses through a well-characterized inflammation to dysplasia to carcinoma sequence. Quantitative estimates of overall CA-CRC risks are highly variable ranging from 2% to 40% depending on IBD severity, duration and location, with IBD duration being the most significant risk factor associated with CA-CRC development. Recently, studies have identified IBD patients with similar patterns of colonic inflammation, but that differ with respect to CA-CRC development, suggesting a role for additional non-inflammatory risk factors in CA-CRC development. One suggestion is that select IBD patients carry polymorphisms in various low penetrance disease susceptibility genes, which predispose them to CA-CRC development, although these loci have proven difficult to identify in human genome-wide association studies. Mouse models of CA-CRC have provided a viable alternative for the discovery, validation and study of individual genes in CA-CRC pathology. In this review, we summarize the current CA-CRC literature with a strong focus on genetic predisposition and highlight an emerging role for mouse models in the search for CA-CRC risk alleles.

Key words: Colitis-associated colorectal cancer; Inflammatory bowel disease; Forward genetics; Susceptibility genes; Azoxymethane; Dextran sulfate sodium; Mouse models

Core tip: Colitis-associated colorectal cancer (CA-CRC)

is the cause of death in 10%-15% of inflammatory bowel disease (IBD) patients. Quantitative estimates of overall CA-CRC risk are highly variable and depend of the severity, duration and location of active IBD. Recently, studies have identified IBD patients with similar patterns of colonic inflammation, but that differ with respect to CA-CRC development, suggesting a role for additional non-inflammatory risk factors in CA-CRC development. In this review, we summarize the current CA-CRC literature with a strong focus on genetic pre-disposition and highlight an emerging role for mouse models in the search for CA-CRC risk alleles.

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INTRODUCTION

Inflammatory bowel disease (IBD) is an umbrella term used to describe chronic-relapsing inflammatory conditions of the intestinal tract[1]. While there are several subtypes of IBD, the two most common are Crohn's disease (CD) and ulcerative colitis (UC). CD is characterized by inflammation throughout the entire gastro-intestinal tract with lesions most commonly found in the small intestine and proximal colon. Approximately 60% of CD patients have colonic involvement, with only 20% having isolated colonic disease[2]. In CD, the inflammation is transmural, traversing multiple layers of the intestine, and typically occurs in patches[1]. In UC, inflammation arises in the rectum and spreads proximally in a continuous manner, rarely extending into the small intestine and is confined to the mucosal layer. Worldwide IBD incidence rates are highly variable (UC: < 1-24.3/100000, CD: 1-20.2/100000), with higher incidence recorded in Western and Northern Europe, Australia and North America and lower incidence in Africa (excluding South Africa), Asia and South America[3-5]. A large-scale meta-analysis of 107 IBD studies (57 CD and 50 UC) recently determined that CD incidence increased in 75% of CD studies and 60% of UC studies over a period of at least 10 years[4]. As IBD patients exhibit a low mortality rate, the global prevalence of IBD is expected to increase in the coming years.

Colitis-associated colorectal cancer (CA-CRC), which develops in areas of active colonic inflammation, is listed as cause of death in 10%-15% of all IBD patients[6,7]. As rates of IBD increase, rates of subsequent CA-CRC are also predicted to increase. From a colon cancer perspective, inflammation is the third most common CRC risk factor, after the hereditary CRC syndromes familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colon cancer (HNPCC). However, unlike FAP and HNPCC, whose etiologies are well characterized, the specific etiologies underlying increased CA-CRC are still being elucidated. In this review, we briefly highlight the current literature with respect to CA-CRC etiology and epidemiology and compare and contrast CA-CRCs relative to non-inflammatory CRC conditions and IBD. In addition, we speculate on a possible function for genetic pre-disposing risk factors in CA-CRC and a role for animal models in elucidating these genetic effects.

EPIDEMIOLOGY, ETIOLOGY AND SURVEILLANCE

CA-CRC is listed as cause of death in 10%-15% of IBD patients[7]. CA-CRC mortality is approximately 50% (CD: 46%, UC: 50%) and this suggests that between 20%-30% of IBD patients will develop CA-CRC within their lifetime[6]. Both UC- and CD-CRC are early-onset conditions presenting with an average age of onset between 40-55 years of age[6,8-10]. UC-CRC is primarily identified in the rectum and sigmoid colon, whereas CD-CRC is more evenly distributed between the right-colon (ascending), sigmoid colon and rectum, although only a small proportion of CD patients have colonic disease[6,11]. The differences with respect to tumor location may reflect differences in location of active IBD as 76% of CD-CRCs and 100% of UC-CRCs arise in areas of macroscopic IBD. CA-CRC patients often present at diagnosis with multiple synchronous carcinomas (CD-CRC: 11%, UC-CRC: 12%) and with lesions showing a high proportion of mucinous and signet ring features (CD-CRC: 29%, UC-CRC: 21%)[6].

According to the American Cancer Society, individuals at increased risk for CA-CRC should undergo routine colonoscopy at 1-2 year intervals starting 8-12 years post-disease diagnosis (www.ccfa.com). It is also recommended that at least four random colonic biopsies be taken for every 10 cm of colon examined during these routine colonoscopies, as approximately 20%-50% of colon dysplasia cannot be detected by visual inspection alone[12,13]. Intraepithelial neoplasms are highly variable with respect to appearance and may present as raised (pedunculated or sessile) or flat (plaque or bump) lesions[14]. Flat lesions are a unique feature to CA-CRC, rarely being detected in familial or sporadic CRC, and are generally associated with high risk of transformation into CA-CRC[15]. The identification of CA-CRC can also be further complicated by large benign inflammatory pseudopolyps, which form during mucosal regeneration and ulcer healing.

Inflammation in CA-CRC pathogenesis

Quantitative estimates of overall CA-CRC risks are highly variable ranging from 2% to 40% depending on IBD severity, duration and location[7]. CD patients with disease isolated to the small intestine only are not at increased risk of CD-CRC supporting the strong

link between inflammation and CA-CRC[6]. CRC risk in UC has been estimated at 2% after 10 years, 8% after 20 years and 18% after 30 years of disease[8]. Studies of UC-CRC have also noted a high concordance between CA-CRC risks with location/extent of disease. For example, Ekbom *et al*[16] identified a standardized incidence ratio (SIR) of 1.7 for proctitis (rectal only), 2.8 for left-sided colitis and 14.8 for pancolitis (defined as extensive colitis, or colitis involving the entire colon).

Studies of CD-CRC are complicated by vast heterogeneity with respect to CD anatomical sites. However, as with UC, CA-CRC risk associations have been correlated with duration/severity of disease. The relative risk (RR) of CD-CRC based on duration of disease was calculated to be 2.9, 5.6 and 8.3 after 10, 20 and 30 years of disease, respectively[17]. In 2007, a CRC meta-analysis by disease site estimated a RR of 0.85, 4.3 and 13.4 for small bowel only, ileocolic and colon CD, respectively[18]. CD-CRC RR is increased to 18.2 in patients with extensive disease.

One of the oldest and most prevalent treatments in IBD is administration of the non-steroidal anti-inflammatory (NSAID) drug 5-aminosalicylic acid (5-ASA) or its derivatives. 5-ASA modulates mucosal inflammation through several mechanisms including: the down regulation cyclooxygenase 2; inhibition of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β); decreased nuclear factor kappa beta (NF-$\kappa\beta$) activation and modulation of peroxisome-proliferator activated receptor gamma (PPAR-γ)[19]. While the protective effects of 5-ASA in IBD are well established, the literature examining 5-ASA as a preventative agent in CA-CRC is controversial. Some studies have demonstrated up to a 97% reduction in CA-CRC risk in patients receiving regular 5-ASA therapy[20-22]. However, recent studies tend to support no protective effects of regular 5-ASA use on CA-CRC risk[23-25]. These discrepancies highlight the complex nature of CA-CRC. It also leads to questions regarding whether there may be certain non-inflammatory factors, such as genetic predisposition that may influence the efficacy of 5-ASA therapeutics.

CA-CRC initiation and progression is dependent on the accumulation of mutations in various tumor suppressors and oncogenes in intestinal epithelial cells[26]. Support for inflammation as a key mediator in CA-CRC pathogenesis comes from animal studies showing increased DNA damage and tumor formation following extended periods of colitis in mice in the absence of a known DNA mutagen[27]. The specific mechanisms through which inflammation regulates CA-CRC initiation and progression are not well understood. It has been suggested that reactive oxygen species (ROS) produced by immune cells during colitis may play a crucial role in promoting DNA damage. Epigenetics, cytokines and the microflora are also thought to be important in mediating cross talk between increased inflammation and CA-CRC and are reviewed in[28].

Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a rare disease characterized by inflammation, fibrosis and subsequent narrowing of the common bile duct. This narrowing leads to the accumulation of bile in the liver resulting in cirrhosis and future liver failure thus reducing life expectancy[29]. There is a strong correlation between IBD and PSC, with approximately 70% (CI: 46.5% to 98.7%) of PSC patients presenting with concomitant IBD, usually in the form of UC[30]. This corresponds to 8% of IBD patients developing coexisting PSC[31,32]. The specific etiology underlying PSC development is complex, but similar to IBD is thought to arise due to a combination of genetic, environmental and microbial risk factors[33,34].

In 2002, a large-scale meta-analysis concluded that PSC patients were at increased risk of developing CA-CRC compared to both IBD patients without PSC and the general population[35]. While there has since been conflicting data concerning CA-CRC in PSC-IBD patients[32], it is generally accepted that PSC is a risk factor associated with CA-CRC development. The explanation behind increased CRC in PSC-IBD patients remains elusive, but may be associated with increased levels of bile acid. Co-diagnosis of IBD and PSC is important to clinicians, as there is some evidence to suggest that treatment with ursodeoxycholic acid (UDCA) may reduce risk of CA-CRC, although additional testing is still necessary[36].

Evidence for non-inflammatory factors in CA-CRC pathogenesis

In addition to strong evidence linking extent and duration of colonic inflammation to CA-CRC risk in IBD patients, there have recently been several observations in humans and mice to suggest a role for non-inflammatory factors in CA-CRC initiation/progression. Family history of CRC development is an important parameter to assess in IBD patients as a positive family history of CRC is associated with a 2-fold greater risk of developing CA-CRC[37]. Studies of human UC and CD-CRC have also shown increased risk of CA-CRC in patients diagnosed with IBD at a young age. For UC-CRC, the absolute CRC risk 35 years post-diagnosis was 40% *vs* 30% in early-onset (age 15 or less) and late-onset UC patients diagnosed with pancolitis, respectively[16]. This was subsequently confirmed in a large scale meta-analysis whereby patients with UC diagnosed prior to 25 years of age were 13 and 70 times more likely to develop CA-CRC compared to older UC patients and the general population, respectively[38]. A similar trend was seen in CD with an increased RR of 21.5 *vs* 1.6 in patients younger and older than 25, respectively and subsequently confirmed in second unrelated CD cohort[18,39]. The specific etiology underlying increased CA-CRC risk in younger onset IBD is still being investigated.

In 2014, Connolly *et al*[40] compiled a cohort of

UC patients to study the role of select IBD genes in CA-CRC. In this study, two cohorts were identified; patients with CA-CRC and those without, despite having similar amounts of UC-inflammation in both cohorts. Similar observations in mice with high levels of colonic inflammation, but low levels of CA-CRC have also been reported and will be discussed further in the mouse model section of this review[41,42]. CA-CRC divergence among individuals with similar IBD status suggests a role for other non-inflammatory factors in mediating CA-CRC initiation or progression and it has been suggested that similar to many other complex traits, select IBD patients are genetically pre-disposed to developing CA-CRC.

GENETIC ASSOCIATIONS IN HUMAN CA-CRC

The "common disease, common variant" hypothesis, stipulates that common complex diseases, such as cancer, diabetes and IBD, arise in part due to common genetic variants (single nucleotide polymorphisms, SNPs) within the genome[43]. To understand the rationale for hypothesizing genetic predisposition in CA-CRC, we must reiterate the similarities with respect to cancer progression between CA-CRC and a type of non-inflammatory CRC, often referred to as familial CRC.

Familial vs colitis-associated colorectal cancers

Genetically, CRCs can be categorized on a sliding scale of pre-disposing risk, which describes the predicted effect size of a given CRC risk variant compared to the minor allele frequency [(MAF), the abundance of the minor allele within a reference population][44]. At one extreme, there are the rare, but well-characterized Mendelian or Hereditary CRC syndromes, such as FAP or HNPCC, whose mutations are associated with a high penetrance of disease symptoms and are easily identified in large families with multiple affected individuals. At the other extreme are familial CRCs, which present with fewer affected individuals per family, and arise, in part, due to common genetic variants within a class of genes known as low penetrance tumor susceptibility genes[45].

In familial CRCs, like most cancers, the balance between cell proliferation, differentiation and apoptosis becomes progressively disrupted through the accumulation of mutations in several signaling pathways encompassing *WNT*, *RAS*, *p53*, *DCC* and *TGF-β* genes. This is referred to as the adenoma-carcinoma sequence progression[46]. Analysis of invasive familial and CA tumors show a similar pattern of acquired molecular alterations and hence CA-CRC was originally categorized as a subtype of familial CRC. This led to speculation that low penetrance tumor susceptibility genes, which are important in familial CRC, could also be important in CA-CRC initiation and progression.

However, the timing and frequency of these genetic events differ between familial and CA-CRC and therefore it has been hypothesized that variants in different genes may be associated with both cancers. For example, mutations/deletions of *p53* are early events in CA-CRC with 50% of ulcerative colitis (UC) patients having *p53* mutations compared to approximately 10% of non-inflammatory adenomas (Figure 1)[28,47]. But *APC* mutations are rare events in CA-CRC (27.5% of high grade dysplasia) compared to 50% in non-CA-CRC adenomas[28,48].

CA-CRCs progress through the colitis-dysplasia-carcinoma sequence associated with the development of inflammation, indefinite, low-grade and high-grade dysplasia, with eventual progression to carcinoma (Figure 1)[26]. Dysplasia describes the abnormal growth and development of colon cells, with indefinite dysplasia describing early changes that cannot be categorized as either negative or positive for dysplasia. It is interesting to note that key inflammatory mediators such as reactive oxygen and nitrogen species (ROS and NOS), as well as chemokines and cytokines (IL-6, STAT3, TNF-α, IL-10, IL-12 and IL-23) all participate to orchestrate the conversion of a normal epithelium to indefinite dysplasia, which again highlights a variable role for inflammation in CA-CRC transformation[28].

Genome-wide association studies

The completion of the Human Genome Project, the International HapMap Project and increased technological power has led to the advent of genome-wide association studies (GWAS)[49]. GWAS compare the prevalence of thousands of common genetic variants [single nucleotide polymorphisms (SNPs)] within healthy (control) and disease (case) cohorts looking for allelic imbalance indicative of disease association[49].

Both IBD and familial CRC have been associated with polymorphisms in low penetrance disease susceptibility genes, with numerous positive associations detected in GWAS. For IBD, more than 200 loci have been identified, the largest number for any common complex disease (http://genome.gov/gwastudies). As IBD pathogenesis is driven by aberrant immune responses against the commensal bacteria of the lumen, it is not surprising that a large number of genes within IBD loci have been associated with epithelial barrier maintenance and permeability, cytokine signaling and pathogen recognition/clearance[50]. Some of the most well characterized genetic associations are *NOD2*, *IL-23R* and *ATG16L1* involved in bacterial sensing, the IL-23 inflammatory response and autophagy, respectively[51]. To date more than 40 loci have been associated with familial CRC (http://genome.gov/gwastudies), with many of the SNPs mapping to regions in strong linkage disequilibrium (LD) with members of the TGF-β signaling pathway,

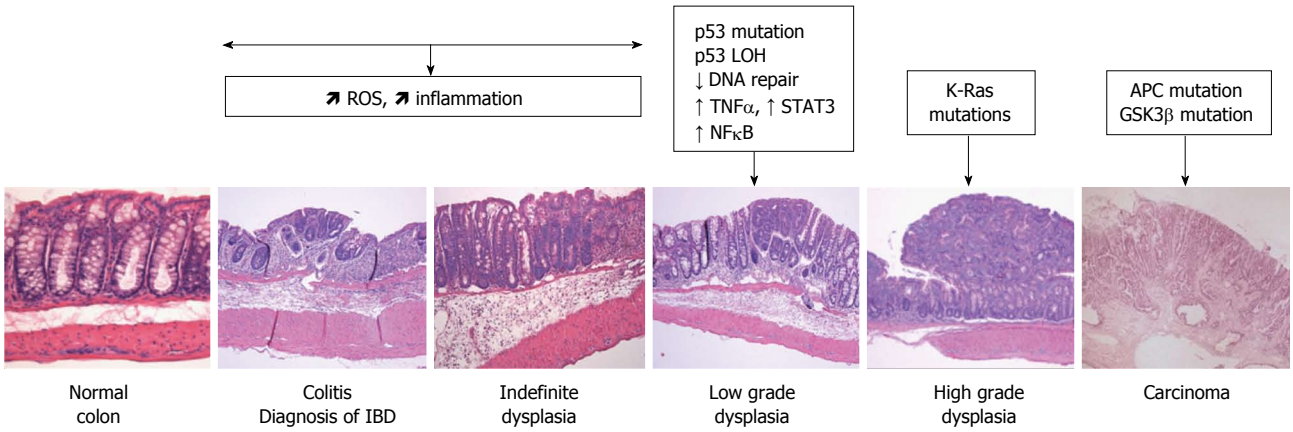

Figure 1 Progression of colitis-associated colorectal cancer. Colitis-associated colorectal cancer progresses through a colitis-dysplasia-carcinoma sequence associated with the development of inflammation, low-grade, high-grade dysplasia and eventually carcinoma due to molecular alterations. IBD: Inflammatory bowel disease; ROS: Reactive oxygen species.

highlighting an important role for TGF-β signaling in CRC[52]. There is little overlap between known IBD and CRC loci in humans suggesting different etiologies to both diseases.

Lack of GWAS for colitis-associated colon cancer

Unlike familial CRC and IBD, there have been very few GWAS performed to identify genetic loci regulating susceptibility to CA-CRC. In part, this may be due to high numbers of IBD patients undergoing colectomy, making identification of IBD patients with and without CA-CRC difficult[53,54]. CA-CRC is influenced by numerous risk factors including age at IBD-onset, and duration/extent of IBD colonic involvement[7,38]. While not essential for early CA-CRC GWAS it may also be important to segregate CA-CRC patients into categories associated with differences with respect to age of diagnosis, ethnicity and extent of inflammation, as different genes may underlie CA-CRC in different colonic microenvironments.

In 2009, the UK IBD Genetics Consortium identified and published a novel UC locus situated on chr. 16 (16q22)[55]. Interestingly, this locus had previously been associated with increased CRC risk[56]. Therefore, it has been speculated that this locus may also play an important role in CA-CRC. However, a recent study showed no association between any known UC loci and UC-CRC risk, disproving this hypothesis[40]. Recently, the STAT3 locus has been associated with both IBD and CA-CRC, exerting its effects in a TP53-dependent manner. This is a promising step in the identification of CA-CRC loci in humans[57].

MOUSE MODELS OF COLITIS-ASSOCIATED COLON CANCER

The complex and heterogeneous genetic component of complex diseases can be difficult to tease apart in human populations due to confounding environmental and lifestyle variables. However, these traits can be dissected in genetically well-defined inbred and recombinant congenic mouse strains[58]. Mice are not particularly prone to the spontaneous development of IBD or CRC and therefore disease induction in mice can be performed using dietary modifications, infectious agents, genetic mutation or chemical reagents[59]. To date more than 100 different mouse models of CRC, IBD, and CA-CRC have been published. For a comprehensive review of these, see[58,60-62].

We have narrowed the focus of this review to three relevant areas; the *Il-10* knockout genetic model of colitis/CA-CRC, the AOM/DSS model of CA-CRC and the mapping of genetic loci regulating susceptibility to CA-CRC using forward genetic approaches.

The Il-10 model of colitis and CA-CRC

Many common colitis and CA-CRC models involve deleting the expression of a specific gene or multiple genes. These models are associated with an increase in IBD (either UC or CD) with or without subsequent CA-CRC[60]. The most-well characterized of these genetic models involves the deletion of the *Il-10* gene encoding a pleiotropic anti-inflammatory cytokine produced by monocytes and lymphocytes that acts to dampen and terminate immune responses[63]. In 1993, Kuhn *et al*[64] generated the 129/B6 *Il-10*$^{-/-}$ knockout mouse line (*Il-10tm1Cgn*). These mice showed a high incidence of weight loss, anemia and enterocolitis 1-3 mo after birth. Enterocolitis was first detected in the proximal colon and then in the remaining colon, the duodenum and the proximal jejunum of the small intestine and mimics human CD, associated with discontinuous, transmural inflammation, ulceration and thickening of the bowel wall[64-66].

Enterocolitis in *Il-10*$^{-/-}$ mice is strain-dependent, suggesting a strong role for genetic factors in disease pathogenesis. The most sensitive genetic backgrounds are C3H/HeJBir and 129/Sv, with 100% of the mice developing severe colitis before 3 mo of age[66,67]. C3H/HeJBir mice, with a wild type *Il-10* gene are also

susceptible to spontaneous colitis[60]. However, CA-CRC susceptibility has not been assessed in the C3H/HeJBir *Il-10*$^{-/-}$ mice[67]. On the 129/Sv background, 67% of the mice develop adenocarcinomas in the first 6 mo of life[66]. As evaluated by histopathology, BALB/cJ *Il-10*$^{-/-}$ mice have a higher incidence of spontaneous colitis (100%) compared to B6 *Il-10*$^{-/-}$ mice (57%) at 3 mo of age, but a lower incidence of colonic tumors (29%) at 6 mo of age compared to 129/Sv *Il-10*$^{-/-}$ mice. B6 *Il-10*$^{-/-}$ mice do not develop colonic adenocarcinomas within this timeframe. NOD/LtJ *Il-10*$^{-/-}$ mice also develop severe colitis, associated with 100% incidence of rectal prolapse, although the time frame for disease development was not specified[68]. These NOD/LtJ *Il-10*$^{-/-}$ mice are not good models for CA-CRC as high incidence of rectal prolapse prevents long-term studies in these mice. Together, these studies highlight an important role for genetic backgrounds in colitis and CA-CRC susceptibility.

Generally, experiments of colitis and CA-CRC in *Il-10*-deficient mice support a strong role for inflammation as the driving factor underlying increased CA-CRC risk. However, an exception to this is a study from Arthur *et al*[69] who demonstrated similar inflammatory profiles in *Il-10*$^{-/-}$ mice infected with *E. faecalis* and *E. coli*, with only the latter being associated with increased CA-CRC, supporting a role for non-inflammatory mediators of CA-CRC.

The AOM/DSS model of CA-CRC

Chemical models of colitis and CA-CRC are advantageous as treatments are relatively inexpensive and easy to administer producing highly reproducible results. These models offer a distinct advantage compared to genetic models as time of onset, duration and severity of colitis/CA-CRC can be adjusted by changing the dose and/or length of the treatment protocol. In addition, unlike genetic models of colitis and CA-CRC, the inflammatory agents can be removed and thus the healing/regeneration process can be studied in detail. In addition, these models highlight a probable role for genetic factors in CA-CRC, with some mice developing high levels of colonic inflammation, yet low levels of CA-CRC and *vice versa*.

In 2003, Tanaka *et al*[70] published results showing that a single azoxymethane (AOM) injection in CD-1 mice, followed a week later by a 7-d dextran sulfate sodium (DSS) treatment, was sufficient to induce macroscopically visible tumors 20 wk post-initiation. Mice treated with only a single AOM or single DSS injection did not develop tumors within this 20-wk period, suggesting that combined administration of AOM and DSS is essential for tumorigenesis. This AOM/DSS protocol has since become one of the most popular models to study the influence of dietary, microbial and genetic factors of CA-CRC progression and initiation[58]. Interestingly, permissive mice given multiple injections of AOM develop CRC, reminiscent of human familial CRC, while those given DSS-alone develop an UC-like phenotype. This allows for common and unique genetic signatures to be identified between the AOM/DSS CA-CRC protocol and the AOM-only CRC and DSS-only IBD protocols.

AOM is a colon specific carcinogen that, when activated, generates a methyl cation that can react with deoxyguanosine at either the N^7 or O^6 position; with the latter leading to the formation of deoxymethylguanosine, resulting in mismatched base pairing and subsequent G to A transitions. DSS is a long chain (5-140 kDa), negatively charged polysaccharide derived from the esterification of dextran and chlorosulfonic acid[71]. When administered to rodents in drinking water, DSS is a highly potent inducer of colitis, mimicking human UC[72]. The location of colitis is highly dependent on the DSS molecular weight, with low weight DSS (5 kDa) inducing lesions in the cecum and proximal colon, mid weight (40 kDa) DSS provoking lesions in the mid and distal colon and high weight DSS (500 kDa) failing to induce colitis in mice[73]. All future mention of DSS refers to mid-weight (approximately 36-54 kDa) DSS.

Inbred strains of mice differ with respect to AOM/DSS-induced CA-CRC susceptibility, with strains such as BALB/c, Swiss Webster, CBA/J, CD1, A/J and FVB/NJ behaving as susceptible and strains such as C3H/HeJ, C57Bl/6 (B6) and DBA/2J being resistant[70,74-78]. Testing for DSS-induced colitis in some strains, such as BALB/c, CBA/J and DBA/2J, suggests that the extent of colonic inflammation is an important driver for CA-CRC[41,72]. However, C3H/HeJ mice are highly susceptible to DSS-induced colitis, yet resistant to CA-CRC, suggesting that inflammation alone does not determine CA-CRC susceptibility[41]. We have also shown that A/J mice, while more susceptible to CA-CRC than B6 mice, develop lower levels of overall colonic inflammation compared to B6 mice following AOM/DSS treatment[78]. Studies of myeloid translocation gene, related 1 (*Mtgr1*) gene deficiency in mice have demonstrated reduced tumor burden following AOM/DSS treatment despite an increased colonic inflammation, again suggesting a role for non-inflammatory, possibly genetic factors in CA-CRC[79].

Recently, Gao *et al*[80] compared global gene expression patterns in untreated BALB/c inbred mice, to those treated with AOM/DSS, AOM-only and DSS-only. As expected, both the DSS- and AOM/DSS-treated mice showed evidence of increased colonic inflammation, which was notably absent in the AOM-only and untreated mice. However, despite the strong influence of inflammation in the AOM/DSS-treated mice, approximately 50% of the identified differentially expressed genes were unique to the AOM/DSS treatment group and were not observed in the AOM or DSS-only groups. Li *et al*[81] also recorded unique genetic signatures association with AOM/DSS-induced CA-CRC, compared to chronic murine colitis,

confirming this observation. Collectively, these studies suggest that CA-CRC susceptibility is associated with unique genetic signatures. Identification of genes specific to CA-CRC may aid in the identification of IBD patients with rapid onset CA-CRC or those who develop CA-CRC despite low levels of colonic inflammation.

Mouse loci identified using forward genetic studies

The identification of genes associated with the development of various complex traits, can be identified using either forward or reverse genetic approaches in mice. Forward genetics is a phenotype-driven approach in which mutations are identified underlying disease traits through the generation of informative mouse crosses followed by linkage analysis[82]. This is the converse of reverse genetics in which a range of phenotypes are characterized for a given genetic mutation[83]. Reverse studies are easier to conduct and are shorter in duration than forward genetic studies, but can be hampered by inefficient knockdown or genetic background effects[83,84]. In addition, forward genetic screens are advantageous as they are conducted without bias as to the types of mutations detected, with mutations mapping to genes that are often unlikely to be tested using reverse genetic approaches and represent a spectrum of mutations more likely to be detected in human disease. Forward genetic studies typically use 4 distinct types of mouse populations; F2, N2, recombinant congenic mice (RCS) or recombinant inbred mice (RI), often using more than one informative population.

Numerous non-inflammatory CRC and IBD susceptibility loci have been mapped in mice using a forward genetics approach and therefore, it is not improbable to hypothesize that CA-CRC loci could be identified using the same approach. These forward genetic approaches are possible as inbred mice differ with respect to susceptibility to all three of the above diseases. Figure 2 summarizes the known IBD, CRC and CA-CRC loci mapped using a forward genetics approach. With respect to IBD, these loci have been mapped using spontaneous (SAMP1/YitFC), chemical (DSS, TNBS), genetic (*Il-10*$^{-/-}$, *Gnai*$^{-/-}$, *Gpx1/2*$^{-/-}$) and infectious (*Helicobacter, Trichuris muris*) models of colitis, while non-inflammatory CRC loci have been mapped using the *Apc*$^{Min+/-}$ mouse model of CRC (mimicking FAP) and AOM (or the AOM precursor dimethylhydrazine)-only models[42,85-101]. Despite differences with respect to strains of mice tested and models of disease induction used, these studies share a common feature, *i.e.,* each cross identified multiple genetic loci regulating susceptibility and each locus controls a small proportion of the phenotypic variation (< 20%).

However, only 3 CA-CRC loci have been identified (Figure 2). The first locus referred to as *Hiccs*, regulates *Helicobacter hepaticus*-induced colitis and CA-CRC susceptibility[88]. This *Helicobacter* model however, is a poor recapitulation of human disease, with mice developing lesions exclusively in the proximal colon.

Our laboratory has also mapped two additional loci that regulate CA-CRC susceptibility. To map these loci, we first defined that A/J mice, contrary to B6 mice, were susceptible to AOM/DSS-induced CA-CRC. Then, using forward genetics and (A/J X B6) F1 and F2 cohorts, we identified and mapped a novel A/J-derived CA-CRC susceptibility locus to mouse chromosome 9, centered around marker D9Mit67. This novel locus was named *Ccs4*[78]. Further analyses of (A/J X B6) F2 mice homozygous for A/J alleles at *Ccs4* identified a second locus on the distal part of mouse chromosome 14 (peak marker rs13482311, 93.5 Mb) that acts to regulate tumor susceptibility in an additive fashion with the *Ccs4* locus. F2 mice homozygous for A/J alleles at both loci (*Ccs4* and chromosome 14) are as susceptible to CA-CRC as the A/J controls, while mice homozygous for B6 alleles are as resistant as the B6 controls, thus supporting the role of two loci in this CA-CRC model. Two locus systems are rarely identified in human GWAS studies, in part due to the low penetrance of the second locus. The ability to detect such interactions in mice provides a framework to search for such associations in humans. In our studies, we also detected higher levels of inflammation in the resistant B6 colons, suggesting that elevated inflammation is not the primary driver of this differential CA-CRC susceptibility. It is interesting to note that an unrelated locus on chromosome 3, namely *Ccs3*, is the primary driver of AOM-induced CRC susceptibility in these same strains, suggesting that these CRC loci may be specific to CA-CRC[102]. The success of this initial genetic screen has led us to hypothesize that other novel genetic factors may also regulate susceptibility to CA-CRC in different inbred mouse strains, which we are currently assessing.

CONCLUSION

CA-CRC is a complex disease arising from a combination of dietary, lifestyle, microbial and genetic factors. In addition, disease risk is tightly correlated with severity, location and duration of colonic inflammation (IBD). CA-CRC risk is increased in early-onset IBD patients and this specific subset of IBD patients is increasing in North America, suggesting that CA-CRC may be a growing concern for future generations[103]. It has been well established that various reverse genetic approaches are ideal for identifying genes associated with increased inflammation and subsequent CA-CRC. However, we have recently shown that we can use forward genetics and the common AOM/DSS model of CA-CRC to identify and map novel loci regulating susceptibility to CA-CRC. By identifying parental strains for mapping, discordant with respect to their colitis and CA-CRC phenotype,

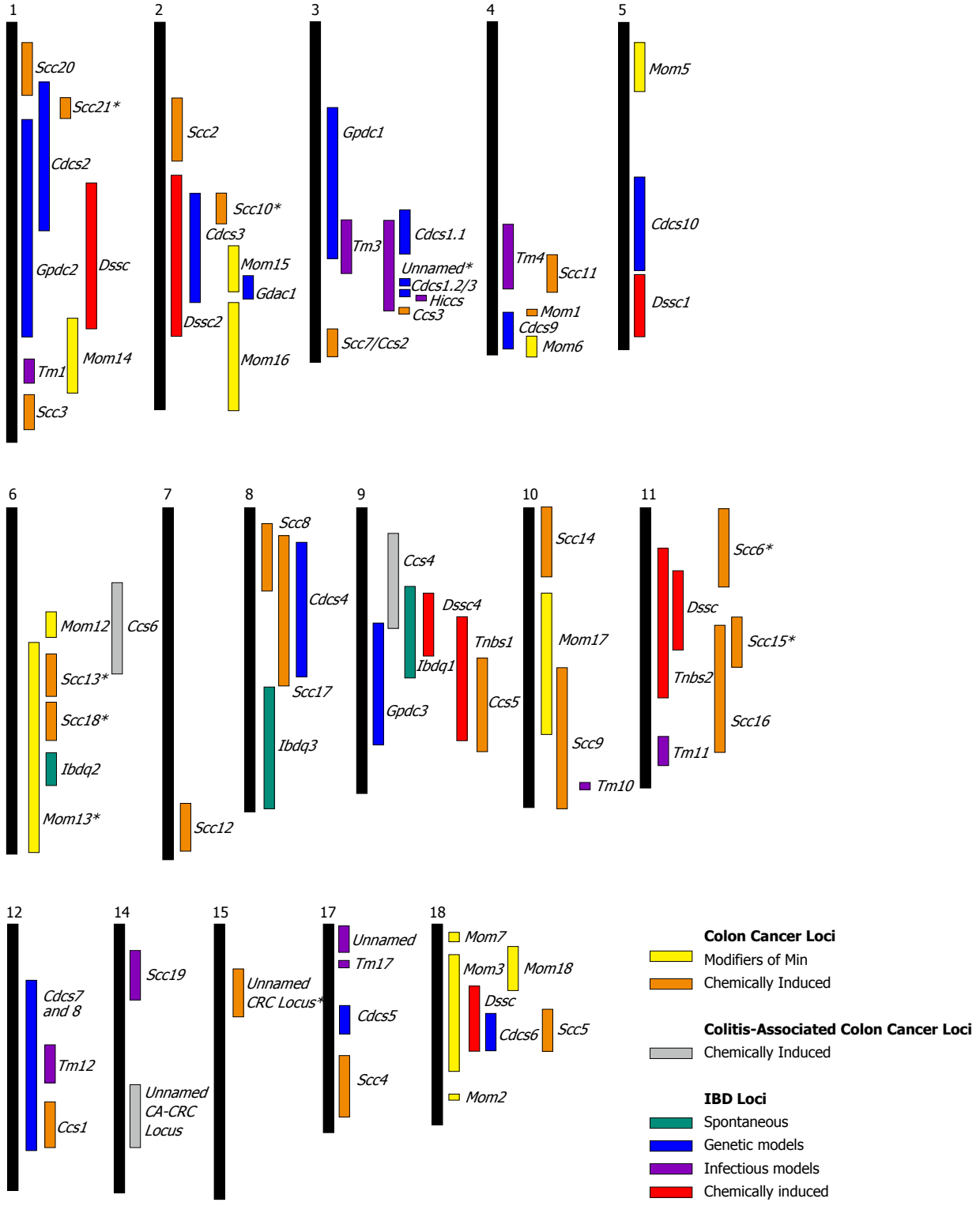

Figure 2 Mouse inflammatory bowel disease and colorectal cancer susceptibility loci. Summary of the current inflammatory bowel disease and colorectal cancer (CRC) loci mapped in inbred mice using forward genetic studies. Arranged by chromosome, each locus has been drawn to scale based on the current mapping data for each. Putative loci or loci that lack mapping data have been excluded. Loci whose precise map location is unknown (indicated with a *) have been drawn centered over the peak marker of association. *Ccs*: Colon cancer susceptibility; *Cdcs*: Cytokine deficiency in colitis (*Il-10*$^{-/-}$ mouse model of colitis); *Dssc*: Dextran sulfate sodium-induced colitis; *Gpdc*: G protein deficient colitis; *Hiccs*: *Helicobacter hepaticus*-induced colitis and associated cancer susceptibility; *Ibdq*: Inflammatory bowel disease quantitative trait loci (Spontaneous *SAMP1/YitFC* model of colitis); *Mom*: Modifier of min (*Apc*$^{Min+/-}$ model of CRC); *Scc*: Susceptibility to colon cancer; *Tm*: *Trishuris muris*-induced colitis; *Tnbs*: Trinitrobenzene sulfonic acid susceptibility.

we can increase the probability of identifying genetic factors specific to CA-CRC and not factors associated with increased colitis. Such loci can then be assessed in human cohorts, with the hope of identifying patients at high risk for colitis to CA-CRC transformation.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Transanal total mesorectal excision: A valid option for rectal cancer?

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Abstract

Low anterior resection can be a challenging operation, especially in obese male patients and in particular after radiotherapy. Transanal total mesorectal excision (TaTME) might offer technical advantages over laparoscopic or open approaches particularly for tumors in the distal third of the rectum. The aim of this article is to review the current experience with TaTME. The limits and future developments are also explored. Although the experience with TaTME is still limited, it might be a promising alternative to laparoscopic TME, especially for difficult cases where laparoscopy is too demanding. The preliminary data on complications and short-term oncological outcomes are good, but also emphasize the importance of careful patient selection. Finally, there is a need for large-scale trials focusing on long-term outcomes and oncological safety before widespread adoption can be recommended.

Key words: Transanal; Bottom up; Transanal minimally invasive surgery; Laparoscopy; Robotic; Outcomes; Rectal cancer; Total mesorectal excision

Core tip: The current literature regarding transanal total mesorectal excision of the rectum (TaTME) is presented. Outcomes are encouraging. TaTME might be a promising alternative to laparoscopic TME, especially for difficult cases where laparoscopy is too demanding. The limitations and future developments are explored.

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INTRODUCTION

Rectal adenocarcinoma remains one of the most common cancers in developed countries[1]. Its surgical management has evolved in parallel over the past century from open to minimally invasive surgery, then from local resection to total mesorectal excision (TME), and from abdominal to transanal approach.

The adoption of TME was a major step towards better oncological outcomes[2], as were more precise definitions of distal and circumferential resection margins (CRM) and minimum number of harvested lymph nodes[3]. Indeed, achieving a good quality of surgery is of paramount importance for rectal resection[4]. The interest to develop better surgical techniques has therefore been continuously growing.

Whilst the safety of laparoscopy has been established in several randomized studies[5-8], Low anterior resection (LAR) can be technically challenging, especially in obese male patients, and in particular after chemoradiotherapy due to scarring and distorsion of anatomical planes. The risk of positive margins has been reported to be significant after open or laparoscopic surgery, particularly for low and anterior rectal tumors[5,9].

In addition, in challenging patients, difficulties in pelvic exposure and limitations of instrumentation can affect not only dissection but also the preservation of autonomic pelvic nerves and the achievement of a restorative procedure[10]. These unsolved problems have led surgical innovators to explore the concept of laparoscopy for low rectal cancers. Whilst some groups have successfully employed the robotic approach to reduce these risks[11,12], there remains a paucity of data regarding the superiority of robotics regarding the oncological outcomes thus far.

Based on this, the concept of "bottom-up" or transanal total mesorectal excision (TaTME) is attractive. Whilst not novel, TaTME has benefited from the previous experience with transabdominal-transanal (TATA) operation[13-15]. Following the developments of naturally orifice transluminal endoscopic surgery (NOTES), transanal endoscopic microsurgery (TEM) and transanal minimally invasive surgery (TAMIS), TaTME has been reported as feasible and safe in several large studies[16-23]. However, the real oncological impact of this technique remains under scrutiny. The aim of this article is to analyse the current experience with TaTME. The limits and future developments are also explored.

TECHNICAL CONSIDERATIONS

TaTME has been developed to overcome the inherent limits of standard approaches, either open or laparoscopic. Indeed, a laparoscopic LAR remains particularly challenging, notably regarding exposure, rectal dissection, and distal cross stapling of the rectum. Starting with dissection from the perineum seems to offer advantages, by avoiding distal cross stapling in a narrow pelvis. The use of laparoscopic staplers in this situation is difficult as multiple staple firings across the low rectum increase potential for anastomotic leak[24].

As mentioned, the concept to start the dissection from the perineum is not new. Indeed, the TATA approach has proven feasible and safe for many years[25]. However, the authors did not use either minimally invasive instruments or a platform for the transanal portion of the TATA procedure. TaTME might therefore have advantages in terms of vision and dissection due to utilisation of CO_2 for insufflation. Overall though, the global aims are the same, namely: to increase the sphincter-saving rate, to reduce positive margins, and to avoid low staple firing.

TaTME, like TATA, has the potential to define the radial and distal margins more clearly. This might be ideal in patients for whom a laparoscopic pelvic dissection is difficult (male, obese, preoperative radiotherapy, tumor located in the lower third of the rectum), carrying a risk of inadequate oncological clearance[19]. With TaTME, distal margin is assessed precisely from the beginning of the procedure. It therefore has the potential to (1) improve resection quality, and therefore clinical outcomes; and (2) decrease the incidence of abdominoperineal resection (APR), thereby improving sphincter preservation rates[26].

From a technical point of view, a transanal purse-string suture below the tumor ensures an adequate oncological distal margin will be achieved[27]. This approach has the advantage of providing excellent visualization even in a narrow pelvis. It could facilitate the dissection of the Denonvilliers fascia minimizing injury to the prostate, seminal vesicles, or vagina. This is especially true in anterior tumors, as they have a high risk of positive CRM. It might also afford more precise autonomic nerve preservation[18].

Currently, the majority of authors still use abdominal assistance. However, a purely transanal approach is feasible, as reported by several groups[28-30]. A recent systematic review found 10% of groups using a purely NOTES approach[16]. To illustrate, Chouillard *et al*[29] performed 62.5% of their cases without abdominal assistance. However, if splenic flexure mobilization is required, abdominal assistance seems appropriate. It can be performed by single port also[31]. The same is true for the creation of a difunctioning ileostomy[32], which is better approached laparoscopically than

Table 1 Transanal total mesorectal excision and peri-operative outcomes (case series with $n \geqslant 5$)

Ref.	Number of patients	ORT	Conversion rate	Complications rate	LOS	Comment
Marks *et al*[25], 2010	79	NA	2.5%	29.9%	5	TATA approach
Tuech *et al*[22], 2015	56	270	7.3%	26%	10	-
Han *et al*[39], 2013	34	151.6	0	6 leaks	9	13 with low rectal tumors, 15 with upper rectal tumors and 6 with sigmoid tumors.
Rouanet *et al*[21], 2013	30	304	7%	Intraop: 10% Postop: 30%	14	Difficult patients (male, high BMI, CRM threatened…)
Muratore *et al*[20], 2015	26	241	NA	26.9%	NA	-
Atallah *et al*[17], 2014	20	243	NA	65%	4.5	-
Buchs *et al*[44], 2015	20	315.3	15%	30%	7	3 benign cases
de Lacy *et al*[18], 2013	20	234	0	20%	6.5	No readmission
Chouillard *et al*[29], 2014	16	265	6.25%	18.8%	10.4%	-
Wolthuis *et al*[43], 2014	14	148	18%	42.9%	8.8	No readmission
Knol *et al*[40], 2015	10	235	0	10%	6	-
Velthuis *et al*[42], 2013	5	175	0	40%	NA	-
Sylla *et al*[41], 2013	5	274.6	0	60%	5.2	

BMI: Body mass index in kg/m^2; ORT: Operative time in minutes; LOS: Length of stay in days; NA: Not available; TATA: Transabdominal-transanal.

transanally. In cases of abdominal assistance, the TaTME technique allows for working simultaneously both from above and below. The operation is then performed in the lithotomy position utilizing a team approach (either metachronously or synchronously). This can have at least one advantage, namely a shorter operating time[19].

The TaTME approach allows for exteriorization of the specimen transanally. However, transanal extraction of the surgical specimen en bloc may not always be possible, particularly in patients with a narrow, deep pelvis, bulky mesentery, and constraints imposed by other pelvic viscera, such as prostatic hypertrophy[33]. When possible, transanal extraction avoids large abdominal extraction incisions and their associated potential complications. A wound protector is advised to minimize the risk of tumor spillage.

INITIAL EXPERIENCE

The use of minimally invasive instruments and new platforms was inspired by NOTES and TEM/TAMIS. The first experience demonstrated in cadaveric models starting in 2007 by Whiteford *et al*[34] was soon followed by others[35,36]. These authors demonstrated the feasibility of the concept, and recognized the critical steps for this procedure. Three years later, the first human clinical case was published[37]. Although the case was well selected (a female with low BMI and a mid-rectum tumor), the proof of concept was established, confirmed shortly thereafter by several case reports and small case series[28,30,38]. More recently, larger series have been published (Table 1)[17,18,20-22,25,29,39-44], confirming their initial experience.

To illustrate, Tuech *et al*[22] recently published a multicentre study, regrouping 56 TaTME patients. They reported very good short-term (Table 1) and pathological outcomes (Table 2). Interestingly, they also reported their oncological outcomes. They found a local recurrence rate of 1.7% at 24 mo. For their entire series, the overall survival rate was 96.4% after a median follow-up of 29 mo. The estimated 5-year disease-free survival rate was 94.2%. Similar oncological findings were reported by Muratore *et al*[20]. These results compared favorably to large TATA series[25].

In another large published series, Rouanet *et al*[21] reported encouraging outcomes in difficult patients (male, 54% overweight or obese, 83.3% CRM threatened according to preoperative MRI, 96.7% with neoadjuvant treatment). Despite this challenging and unfavourable population, they showed good peri-operative and pathological outcomes. Of note, two cases of urethral injury were observed at the beginning of the experience, emphasizing the need for a significant learning curve and great caution when performing dissection anteriorly. Finally, the overall survival and disease-free survival rates were 80.5% and 88.9% at 24 mo, in a high-risk population.

When assessing a new surgical technique for rectal cancer, the pathological outcomes are of paramount importance. A good quality TME specimen is essential, as it remains an independent risk factor for local recurrence[45]. The majority of authors using TaTME have reported excellent specimen quality and adequate margins (Table 2).

It is quite clear that TaTME, regardless of the specific equipment utilized, the performance of a sequential or synchronous technique, the height of the tumor, or the use of neoadjuvant therapy, seems to influence the ability to achieve a complete or near-complete TME[4], as confirmed in recent systematic reviews[3,16].

COMPARISON TO STANDARD TME

A logical next step was the comparison to standard approaches. Recently, several studies evaluating

Table 2 Transanal total mesorectal excision and pathological outcomes (case series with $n \geq 5$)

Ref.	TME quality	Positive CRM	Distal margins	LN
Marks *et al*[25]	NA	6.3%	1.9 cm	11.4
Tuech *et al*[22]	84% intact and 16% nearly intact	5.4%	1 cm	12
Han *et al*[39]	NA	0%	2.43	12.9
Rouanet *et al*[21]	100% good	13.3%	0.9 cm	13
Muratore *et al*[20]	88.5% complete	0%	1.9 cm	10
Atallah *et al*[17]	89.5% complete or nearly complete	5%	5% positive	22.5
Buchs *et al*[44]	94.1% intact and 5.9% nearly intact	5.9%	2.14 cm	23.2
De Lacy *et al*[18]	100% satisfactory	0%	2.6 cm	15.9
Chouillard *et al*[29]	100% intact	0%	3.6 cm	21
Wolthuis *et al*[43]	NA	NA	NA	NA
Knol *et al*[40]	90% intact	0%	1.94 cm	10.5
Velthuis *et al*[42]	100% intact	0%	0 positive	12
Sylla *et al*[41]	100% intact	0%	0 positive	33

TME: Total mesorectal excision; CRM: Circumferential resection margins; LN: Lymph nodes; NA: Not available.

Table 3 Comparative studies and peri-operative outcomes

Ref.	Number of patients	ORT	Conversion rate	Complications rate	LOS	Readmission
Denost *et al*[46], 2014	50 lap TME	263	10%	44%	8	NR
	50 Perineal TaTME[1]	240	4%	32%	7	
Fernandez-Hevia *et al*[19], 2015	37 lap TME	252	0	51%	9	22%
	37 TaTME	215	0	32%	6.8	6%
Velthuis *et al*[23], 2014	25 lap TME	NR	NR	NR	NR	NR
	25 TaTME					

[1]Perineal dissection. No use of laparoscopic instruments. Lap: Laparoscopy; TaTME: Transanal total mesorectal excision; ORT: Operative time in minutes; LOS: Length of stay in days; NR: Not reported.

Table 4 Comparative studies and pathological outcomes

Ref.	Group	TME quality	Positive CRM	Distal margins	LN
Denost *et al*[46]	Lap TME	62% complete	18%	1 cm	17
	Perineal TaTME[1]	70% complete	4%	1 cm	17
Fernandez-Hevia *et al*[19]	Lap TME	94.6% complete	0	1.7 cm	14.7
	TaTME	91.9% complete	0	2.8 cm	14.3
Velthuis *et al*[23]	Lap TME	72% complete	8%	2.5 cm	13
	TaTME	96% complete	4%	2.3 cm	14

[1]Perineal dissection. No use of laparoscopic instruments. Lap: Laparoscopy; TaTME: Transanal total mesorectal excision; CRM: Circumferential resection margins; LN: Lymph nodes.

TaTME in comparison to laparoscopic TME have been published (Tables 3 and 4).

Lately, Denost *et al*[46] published a randomized trial, comparing standard laparoscopic TME with perineal transanal TME for low rectal cancer (< 6 cm from the anal verge). In contrast to other groups, they performed the perineal dissection using traditional instruments rather than laparoscopic instruments. While they recognized that TEM equipment was an option, they did not need special platforms. Although it was not sensu stricto a TaTME, this experience confirmed the proof of concept, demonstrating that starting from the perineum has some advantages. They showed that the positive CRM rate was reduced in the perineal group (4% *vs* 18% for standard TME; $P = 0.025$). After multivariate analysis, the abdominal dissection was the only independent factor of positive CRM. Furthermore, the quality of the TME specimen was similar in both groups. In addition, the rate of anastomotic leakage (2% *vs* 10%), the operative time (240 min *vs* 263 min) and the conversion rate (4% *vs* 10%) were decreased in the perineal group compared with the abdominal group. These differences did not reach the level of statistical significance.

In a recent case-matched series, Fernández-Hevia *et al*[19] proffered interesting results. They compared 37 laparoscopic TME resections with 37 transanal endoscopic TME resections. Overall they showed better short-term outcomes following TaTME, with a shorter operative time (minus 37 min; $P < 0.01$), a shorter hospital stay (minus 2.2 d; $P = 0.1$), and less readmission (6% *vs* 22% for standard TME; *P*

Table 5 Robotic transanal total mesorectal excision

Ref.	Number of patients	ORT	Complications	LOS	Comments
Huscher *et al*[51], 2015	7	165.7	1 rectal bleeding, requiring blood transfusion	4.8	Negative margins, 6 complete and one nearly complete TME
Gomez Ruiz *et al*[50], 2015	5	398	1 anastomotic leak	6	Negative margins, complete TME
Atallah *et al*[48], 2014	3	376	Pulmonary embolism, stoma high output	4.3	All complete or nearly complete TME. Negative margins
Atallah *et al*[49], 2013	1	381	0	3	Negative margins, nearly complete TME
Verheijen *et al*[52], 2014	1	205	0	3	Negative margins, complete TME

ORT: Operative time in minutes; LOS: Length of stay in days.

= 0.03). The TaTME group tended to present less complications as well (32% *vs* 51%), although this did not reach statistical significance (P = 0.16). Regarding anastomotic leak, there was a trend in favour of TaTME group (5% *vs* 11%, P = 0.39). Finally, with the exception of a longer distal margin (overall + 1.1 cm; P < 0.01), the transanal group showed similar pathological data.

In further case-matched study, Velthuis *et al*[23] focused on pathological outcomes. They showed some advantages for the TaTME approach with 96% of patients having a complete TME specimen, while in the laparoscopic group, only 72% presented an intact specimen (P < 0.05). The difference is even more obvious when considering abdominoperineal excision (83% *vs* 33%). There were less positive CRMs in the TaTME group (4% *vs* 8%), although the difference was not statistically significant.

Overall achievement of oncological resection principles is confirmed by an identical number of lymph nodes harvested in both groups and by similar, if not better, R0 rate after TaTME (Table 4). The same is reported for the quality of TME. Better short-term outcomes might also be expected. So far however, the differences have not reached statistical significance. This could be accounted for by small sample sizes. Nonetheless, these results are promising and should motivate further research.

ROLE OF ROBOTICS

One feature robotic technology offers the surgeon is a 3-dimensional (3D) view. It is thought that this could provide advantages in terms of more accuracy during dissection. Others have also reported the use of 3D laparoscopic camera with success[19]. However, beyond the quality of vision, the interest of robotics is more associated with the manoeuvrability of the instruments and the stability of the platform. After initial successful cadaveric experience[47], published data regarding the clinical use of robotics for TaTME are encouraging albeit limited (Table 5)[48-52]. Of note, the use of robotic technology in this situation might restrict the possibility to work simultaneously from the abdomen and the perineum (concept of a two-team approach), which might have been a source of time saving. Regarding this experience, the feasibility has been established. Although the number of patients remains limited, the safety seems to be similar as standard TaTME. Real advantages are still hypothetical but robotic technology might help to overcome the steep learning curve, which seems to be associated with TaTME. New single-site surgery platforms are awaited. They may facilitate docking and transanal access[53].

LIMITS AND FUTHER DEVELOPMENTS

New technique, new complications?

When any new surgical technique is adopted, safety is of paramount importance. Whilst an increase in complication rates could be anticipated at the beginning of the learning curve, the global safety has to be guaranteed. However, as it was previously shown for other procedures or technique[54,55], the risk of encountering new or unexpected types of complications is real. Whilst the safety profile of TaTME seems at least similar to the standard approach, the risk of local abscess or collection formation needs to be emphasized. Indeed, Velthuis *et al*[56] found a positive pelvic culture in 39% of patients during TaTME. Of these, four (44%) developed presacral abscesses. The remainder of the cultures were negative with none of these patients developing infectious complications. On the other hand, pelvic collection (or anastomotic leak) does not seem to be over-reported in the current literature. Meticulous washout is therefore advocated before and during the procedure, especially before the rectotomy.

One of the most common complications reported was urinary retention and transient urinary dysfunction. Sylla *et al*[41] found 2 patients with urinary dysfunction (40% in their pilot study). Urodynamic testing one month postoperatively demonstrated minimal detrusor activity consistent with a neurogenic bladder. These data were confirmed on a smaller scale by Tuech *et al*[22]; 5 patients presented transient urinary dysfunction (8.9%). This was corrected at 3 mo. On the other hand, in their randomized study, Denost *et al*[46] did not find any statistical differences between perineal and abdominal dissection in terms

of urological complications (6% *vs* 10%, $P = 0.715$). It is therefore worthwhile mentioning the risk of urethral lesions[21] as TaTME may result in an increased incidence of urethral injury, especially at the level of the post-prostatic urethra and particularly in the setting of anterior tumors, and prior pelvic irradiation[26]. It is worthwhile mentioning that this complication rarely, if ever, occurs for standard TME.

Finally, pneumopelvis is worthy of mention, as an aid during dissection. Atallah[26] has noted that CO_2 might also show areolar planes beyond the scope of dissection thus leading the surgeon astray. This could occur in two distinct areas: (1) laterally, at the level of the mid rectum; and (2) posteriorly, at the level of the mid and upper rectum, placing the operating surgeon in a plane that is "too deep", thereby entering the pre-sacral space. Going off plane can result in inadvertent injury to both pelvic sidewall autonomic nerves and the sacral venous plexus posteriorly, resulting in haemorrhage[26].

Oncological outcomes

Oncological outcomes for TaTME are scarcely reported. Preliminary data seem encouraging though. Indeed, in one of the largest series of 56 consecutive patients, Tuech *et al*[22] found a local recurrence rate of 1.7% at 24 mo. After a median follow-up of 29 mo, the overall survival rate was 96.4%. The estimated 5-year disease-free survival rate was 94.2%. These results were substantiated by Muratore *et al*[20], showing an overall survival and disease-free rate of 92.3% after a mean follow up of 21 mo. In addition, they did not report any local recurrence. Even when assessing high-risk patients, Rouanet *et al*[21] found an overall survival and disease-free survival rate of 80.5% and 88.9% at 24 mo respectively.

A word of caution though: the risk of poor outcomes should be mentioned, especially when dealing with locally advanced tumors. In their series, Rouanet *et al*[21] have dealt with 23% of patients presenting an initial T4 tumor. In these circumstances, there is a significant risk of worse pathological and oncological outcomes. The most recent studies[19,44,46] have reported a low rate of preoperative T4 patients. For these challenging patients, it is still not clear which approach is the most appropriate.

Finally, long-term follow-up is required before drawing definitive conclusions regarding the oncological safety of TaTME. Preliminary data are promising though, and at least as good as the standard approach[8,57].

Functional outcomes

In tandem with oncological safety, the issue of functional outcomes should be addressed. Poor function can be attributed to a combination of factors: the increased rate of coloanal anastomosis, partial sacrifice of the internal anal sphincter, and the anal stretch during TaTME. To date, functional outcomes have been poorly investigated but TME experience may yield some clues. Indeed, at least one third of the TME patients might present some degree of temporary incontinence[58]. On the other hand, the extrapolation of these results to TaTME is hypothetical, especially in a population where the rectum has been removed.

Rouanet *et al*[21] showed that at 12 mo after stoma closure, 40% of patients were fully continent, 15% reported incontinence to liquids, 35% to gas, and 25% had stool fragmentation. Atallah *et al*[17] confirmed these results and showed that most of the patients had mild fecal incontinence 8 wk after ileostomy closure. Only one patient presented a life-style-limiting incontinence. In their large multicenter study, Tuech *et al*[22] found 3 patients (5.7%) requiring definitive colostomy because of severe fecal incontinence after inter-sphincteric resection with coloanal anastomosis. In addition, 28% of their studied group reported stool fragmentation and difficult evacuation.

Finally, in sexually active patients, this French group found 66.6% patients with unchanged ejaculation and 11.2% with failure to ejaculate. Impotence was reported in 11.2% of males[22]. These data are in accordance with the standard approach[59,60].

What next?

While promising, it is imperative to raise a note of caution: clearly, only high-volume centres with technically adept, minimally invasive surgeons can produce these results[4]. There is a need to continue to develop and collaborate in an international registry, collecting relevant and high quality data on transanal rectal resection surgery for benign and malignant pathology. This will allow for safe and responsible introduction of a new technology. It may also be a driver for further research and multicentre studies[10]. Recently, the TaTME registry was launched. It is a voluntary database with online access through the LOREC (Low Rectal Cancer Development Program) portal (http://www.lorec.nhs.uk)[10].

Currently, the main open questions can be categorized as follow: (1) How to overcome the technical limitations? (2) Who are the best candidates (selection criteria)? (3) What are the long-term outcomes (oncological and functional)? (4) How to teach this technique? (5) What are the pre-requisite skills for the surgeon? What is the learning curve? (6) What are the associated costs? and (7) Should everyone be doing it (*i.e.*, is there a minimum case volume)?

From a technical point of view, the current platforms are not ideal and relatively unstable. The introduction of Airseal (SurgiQuest) might help to overcome two technical problems: (1) excessive plumes of smoke which obscure the operative field of view; and (2) "bellowing" or collapse and re-expansion of the pelvis with the cycling of CO_2[26]. The experience with TAMIS was encouraging, allowing maintenance of a stable pneumopelvis[61]. However, this technology has a cost and no comparative studies are currently available de-

monstrating clear objective advantages over standard platforms.

As for laparoscopic LAR, the assessment of CRM is still challenging during TaTME. Developments of intra-operative navigation and augmented reality are both new and interesting fields. Recently, stereotactic navigation has been tested for TaTME[62], to ensure R0 resection. This might be particularly relevant for locally advanced tumors. The accuracy was reported to be ± 4 mm. This technology seems to have potential, especially when applied to pelvic and fixed abdominal organs[63], as it was reported for liver surgery[64,65].

A fully NOTES procedure might be the final step. It has already been reported as feasible and safe by others[28-30]. The main technical advantage of NOTES is the absence of abdominal scars, conferring a cosmetic benefit. In addition, a reduction in pain and incision-related complications might be expected too. This said, the splenic flexure mobilization and the stoma formation are probably best performed by an abdominal approach.

The question of selection criteria is probably the most crucial and will continue to animate debate. Even in very difficult patients, Rouanet *et al*[21] showed comparable results. Although the risk of positive CRM was slightly higher than expected (13.3%), it is worthwhile noting that they are still comparable to previous data (COLOR Ⅱ study: 9%-22%)[5,8].

According to Atallah, the best suited surgical candidates are those[33]: (1) considered difficult to approach from above; and (2) who have a distal rectal tumor, and who are not candidates for local excision.

Several local anatomical and pathological factors may also favour TaTME. These include male gender, locally advanced rectal cancer, tumors in the distal third of rectum, narrow and/or deep pelvis, visceral obesity, prostatic hypertrophy, large tumor diameter, and distorted tissue planes due to neoadjuvant radiotherapy[33]. On the other hand, at least at the beginning of the experience, a locally advanced tumor should be avoided.

Of note, this technique can also be utilized for benign disease, particularly at the beginning of the learning curve. Examples include completion proctectomy for ulcerative colitis or complicated rectovaginal fistulae.

As mentioned, long-term (oncological and functional) outcomes are awaited. There remains little information regarding the ileostomy closure rate and the occurrence of late anastomotic strictures[16]. In addition, while preliminary experience of TaTME in comparison to standard LAR is promising, data remains scarce. There is still a clear need for an RCT, and more multicenter series. Again, the need for an international registry is reiterating.

There is definitively a learning curve. It may be steep. Whilst extensive experience with TEM/TAMIS and LAR is a prerequisite, there are no data evaluating this learning curve so far. As for robotic surgery[66], there is a gap between the will to teach a specific technique and the practical aspects to integrate this new training in the curriculum. Many advocate animal and/or cadaveric training prior to attempting the procedure[16]. Dedicated courses need to be developed. Our preliminary experience with hands on cadaver courses has been encouraging, allowing trainees to perform several successful TaTMEs. Finally, mentoring might also form part of the curriculum.

The cost effectiveness of this new technique is unknown. The direct costs might be higher than the standard approach for variety of reasons: a 2-team procedure requires more staff, more equipment, and personnel familiar with (and trained to use) new devices. However, if the short- then long-term outcomes are confirmed to be better after TaTME, the indirect costs could be in favour of TaTME. This assertion currently remains hypothetical requiring larger dedicated studies.

CONCLUSION

Although the experience with TaTME remains limited, it presents a promising alternative to laparoscopic TME, especially for difficult cases where laparoscopy is too demanding. The preliminary data on complications and short-term oncological outcomes are good. They also emphasize the importance of careful patient selection. Finally, there is a need for large-scale trials focusing on long-term outcomes and oncological safety, before widespread adoption can be recommended.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Non-coding landscapes of colorectal cancer

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Abstract

For two decades Vogelstein's model has been the paradigm for describing the sequence of molecular changes within protein-coding genes that would lead to overt colorectal cancer (CRC). This model is now too simplistic in the light of recent studies, which have shown that our genome is pervasively transcribed in RNAs other than mRNAs, denominated non-coding RNAs (ncRNAs). The discovery that mutations in genes encoding these RNAs [*i.e.*, microRNAs (miRNAs), long non-coding RNAs, and circular RNAs] are causally involved in cancer phenotypes has profoundly modified our vision of tumour molecular genetics and pathobiology. By exploiting a wide range of different mechanisms, ncRNAs control fundamental cellular processes, such as proliferation, differentiation, migration, angiogenesis and apoptosis: these data have also confirmed their role as oncogenes or tumor suppressors in cancer development and progression. The existence of a sophisticated RNA-based regulatory system, which dictates the correct functioning of protein-coding networks, has relevant biological and biomedical consequences. Different miRNAs involved in neoplastic and degenerative diseases exhibit potential predictive and prognostic properties. Furthermore, the key roles of ncRNAs make them very attractive targets for innovative therapeutic approaches. Several recent reports have shown that ncRNAs can be secreted by cells into the extracellular environment (*i.e.*, blood and other body fluids): this suggests the existence of extracellular signalling mechanisms, which may be exploited by cells in physiology and pathology. In this review, we will summarize the most relevant issues on the involvement of cellular and extracellular ncRNAs in disease. We will then specifically describe their involvement in CRC pathobiology and their translational applications to CRC diagnosis, prognosis and therapy.

Key words: Colorectal cancer; MicroRNA; Long non-coding RNAs; Circular RNAs; Diagnosis; Prognosis; Therapy

Core tip: For many decades the predominant view of molecular functioning of organisms stated that proteins represent the main regulators of genomes and their dysfunctions were the first cause of diseases. This protein-centred view was too simplistic to explain the complexity of cancer. In the last few years many studies have revealed that about 85% of our genome is pervasively transcribed, mainly as non-protein-coding RNAs (ncRNAs). The discovery of countless molecular alterations of ncRNAs related to cancer changed the paradigms of cancer biology. In this review, we report recent advances in the discovery of ncRNAs involved in Colorectal Cancer pathobiologies, and their potential applications in diagnosis, prognosis and therapy.

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INTRODUCTION

Although Jacob and Monod[1] had suggested in 1961 the centrality of RNA in the flow of genetic information, for many decades the most predominant view remained that proteins represent the main regulatory components of the genome. This "protein-centred" view is indeed simplistic and may be misleading when applied to higher organisms. Recent studies have suggested that there are about 20000 protein-coding genes in the human genome: this is very close to the number of protein-coding genes in *C. elegans*[2,3]. Such observations suggest that these genes alone are not sufficient to appropriately explain the complexity of higher eukaryotes such as mammals and primates[4,5]. An analogous remark may be made on the model proposed by Fearon and Vogelstein, which describes colorectal cancer (CRC) pathogenesis as a sequence of mutations in protein-coding genes: this model has been the paradigm of CRC pathological evolution and has provided a framework for many other cancer studies[6-8]. However, over the years many observations have shown that this model is not able to recapitulate the complexity and heterogeneity of CRC (*in vitro*, but especially *in vivo*)[9,10]. Recent high-throughput studies of the human transcriptome have revealed that about 85%-90% of our genome is dynamically and pervasively transcribed, mostly as non-protein-coding RNAs (ncRNAs)[5,11,12]. In the last decade, many observations have convincingly suggested that ncRNAs significantly contribute to the complex molecular signalling needed to regulate structures and functions in different cells and developmental contexts[13,14]. Accordingly, their dysregulation strongly contributes to the onset and progression of many pathological conditions[15-17]. The discovery of molecular alterations of ncRNAs, related to neoplastic phenotypes, has initiated a shift in the paradigms of cancer biology and has profoundly influenced our understanding of tumour genetics[18-20]. Moreover, several reports have shown that ncRNAs can be secreted by cancer cells into biological fluids, potentially spreading oncogenic signals to other cells: this suggests that cancers may exploit RNA-based, hormone-like mechanisms to advantageously mold their extracellular environment[21,22]. In this review, we will describe recent advances in the discovery of the involvement of ncRNAs in CRC pathobiology, analyzing the contribution of different species of ncRNAs (both cellular and extracellular), their participation to CRC progression and dissemination, and their applications in diagnosis, prognosis and therapy.

NON-CODING RNAS: MANY SIZES AND FEATURES TO PERFORM MULTIPLE FUNCTIONS

It is not precisely known how many ncRNA genes are present in the human genome. ncRNA genes are difficult to identify because of their structural heterogeneity: (1) extreme length variation from 20 nucleotides to > 100 kb; (2) absence of Open Reading Frames (ORFs); (3) no or low evolutionary conservation in many cases; (4) no preferential localization within the genome; and (5) relative tolerance to point mutations[23,24]. The most common (and approximate) classification of ncRNAs is based on their length[25,26]. It divides them into two classes: (1) long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides (nt); and (2) small non-coding RNAs, whose length is equal to or less than 200 nt [*i.e.*, microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs). Other classifications have been proposed to categorize ncRNAs. They can be divided into two classes according to functional features: (1) housekeeping ncRNAs; and (2) regulatory ncRNAs. Housekeeping ncRNAs are constitutively expressed in all cells for their physiological functioning: they include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and telomerase RNAs[27,28]. On the other hand, regulatory ncRNAs can be expressed in a cell-specific way, or during defined stages of development and cell differentiation, and finally in response to external *stimuli*[29,30]. This category comprises miRNAs, siRNAs, lncRNAs (*i.e.*, the RNA molecules more closely involved in cancer biology), and piRNAs[31,32]. It is remarkable that many ncRNAs share features that could allow their assignment to multiple categories, thus eluding systematic classification (for instance: trans-spliced transcripts encompassing huge genomic regions)[33].

SMALL NON-CODING RNAs

This class of ncRNAs includes different types of molecules involved in different steps of RNA synthesis, processing, translation, as well as modulation of transcription initiation [*i.e*., piRNAs, promoter-associated small RNAs (PASRs)], RNA degradation or protein synthesis block (*e.g*., miRNAs, siRNAs), RNA maturation (*e.g*., snoRNAs)[34,35]. Among these RNAs, miRNAs represent the most studied class of ncRNAs: they have also been shown to be tightly associated with neoplastic phenotypes, especially CRC[36,37].

MiRNAs

Originally discovered by Victor Ambros in *Caenorhabditis elegans*, miRNAs are 18-25 nucleotides long, evolutionary conserved, single-stranded RNAs[38]. They are processed from larger precursors through sequential cleavage by two RNase Ⅲ-like enzymes: *Drosha* (in the nucleus) and *Dicer* (in the cytoplasm). By interacting with the protein Ago2, one strand of the resulting duplex can associate with the RNA-induced silencing complex (RISC). In most cases, these miRNAs-RISC complexes target specific mRNA molecules binding to their 3' untranslated regions (UTRs), which may lead to translational repression or cleavage of the mRNAs[39,40]. The former effect may be due to interference with mRNA cap recognition, inhibition of mRNA interaction with the ribosomal subunit during translation initiation, or increased rate of ribosome drop-off during elongation. Degradation is instead mediated by mRNA decapping and deadenylation[41,42]. Currently, over 2000 human miRNAs have been identified by cloning and sequencing approaches. It is predicted that miRNA genes account for 1%-2% of the human genome and control the expression of at least 50% of all protein-coding genes[43]. MiRNAs regulate fundamental cellular processes, such as cell proliferation, differentiation, migration, angiogenesis and apoptosis: accordingly, they are considered potential oncogenes or tumor suppressors in cancer development and progression[44,45]. The discovery of miRNA dysregulation or mutations, etiopathogenetically related to neoplastic phenotypes, has provided new perspectives for the study of complex gene regulatory networks in CRC and other tumours[46-48].

LONG NON-CODING RNAs

Long non-coding RNAs are the broadest and most heterogeneous class of non-protein-coding RNAs: their length is greater than 200 nucleotides, frequently reaching up to 100 kb. They include transcripts that may: (1) be located in intergenic regions [long intergenic non-coding RNAs (lincRNAs)][49]; (2) lie within introns of protein coding genes[50]; (3) partially overlap UTRs or promoters of protein coding genes[51,52]; (4) be transcribed from pseudogenes and control the expression of their protein-coding functional paralogs[53]; and (5) be transcribed ultra-conserved regions (tUCRs), which are highly evolutionarily conserved and may be located in intra- or in intergenic regions[54]. Several thousand putative lncRNAs have been already identified and shown to be expressed in a developmental and tissue-specific manner[55,56]. Recent results have convincingly suggested the involvement of lncRNAs in a wide spectrum of biological processes, such as cell-cycle regulation, stemness, differentiation, and apoptosis[57-59]. Unlike miRNAs, which repress gene expression through a common mechanism involving RISC complexes, lncRNAs exhibit a broad range of mechanisms of action through which they are involved in the modulation of epigenetic regulation, alternative splicing, and protein localization and activity. It is probable that these functions are due to the ability of lncRNAs to bind DNA, other RNAs and proteins. LncRNAs can also serve as decoys, which preclude the access to the DNA of regulatory proteins and prevent transcription of specific genes (*e.g*., lncRNA Gas5, DHFR)[60,61]. Many lncRNAs are associated with polycomb repressive complex-2 (PRC2) or other chromatin-modifying complexes, which modulate epigenetic silencing of target genes (*e.g*., HOTAIR)[62,63]. LncRNAs can serve as scaffolds to bring two or more proteins into functional complexes (*i.e*., telomerase RNA TERC)[64], or are required to properly localize protein complexes[65]. A subset of ncRNAs, named Telomere-associated ncRNAs (telomeric repeat-containing RNA, TERRA), negatively regulates telomere length presumably by inhibiting telomerase activity[66]. LncRNAs may be processed into smaller ncRNAs (*e.g*., MALAT1)[67]; pseudogene transcripts can be processed into siRNAs that regulate protein coding genes through RNA interference (RNAi)[68]. By targeting RNAs through direct sequence complementarity, lncRNAs may also operate as antisense molecules against their targets and modulate alternative splicing events (*e.g*., antisense of ZEB2), or increase the stability of mRNAs by hiding their miRNA binding sites (*e.g*., BACEAS)[51,69]. Circular RNAs (circRNAs) belong to an odd, but extremely interesting class of lncRNA molecules, which has been recently described. CircRNAs can act as natural miRNA sponges to lower miRNA levels: accordingly, they perform a critical role modulating the connection between genotype and molecular phenotype[70]. Dysregulation of lncRNAs has been documented for many complex human diseases, including cancer. Dozens of lncRNAs have been reported to have altered expression in neoplasia and to be controlled by specific oncogenic and tumor suppressor pathways[71,72]. These observations strongly suggest that lncRNAs could be added to the list of proto-oncogenes and tumor suppressors, as suspects potentially involved in oncogenesis. Accordingly, they might also be considered as potential biomarkers and targets for novel therapeutic approaches in neoplastic diseases.

MULTIFACETED NON-CODING RNA LAYERS CONTROL THE COLORECTAL CANCER GENOME

MiRNAs perform key roles in CRC initiation and evolution

Since the original discovery connecting miRNAs to Chronic Lymphocytic Leukemia[73], researchers have convincingly demonstrated that miRNAs play a critical role in cancer. MiRNA oncogenic activity can be tissue-specific. Altered miRNA expression plays an etiological role in the initiation and progression of colon cancer: global miRNA expression patterns can discriminate between normal tissues and CRC tissues more efficiently than mRNA expression patterns. Furthermore, several investigations have shown the ability of miRNA expression patterns to improve diagnosis of poorly differentiated tumours and predict prognosis in CRC (see next paragraph in this review). In 2003, Michael *et al*[74] published the first study on miRNAs in CRC: they identified miR-143 and miR-145 as novel dysregulated miRNAs in colon cancer. Since then, the literature on miRNAs in CRC has grown considerably. This paragraph will provide an overview of the etiological connection between the molecular functions of miRNAs and CRC pathobiology. The first discovered CRC-related miRNAs, miR-143 and miR-145, act as tumor suppressor genes and are downregulated in CRC compared with normal colonocytes[74,75]. miR-143 targets KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) and MACC1 (metastasis-associated in colon cancer-1), thus playing an important role in the regulation of EGFR (epidermal growth factor receptor) and HGFR (hepatocyte growth factor receptor) signalling[76,77]. Both miR-143 and miR-145 modulate CRC cell proliferation: transfection of DLD-1 and SW480 cells with premiR-143 and premiR-145 reduced cell proliferation[78]. MiR-145 indirectly promotes angiogenesis by binding p70S6K1 mRNA and inhibiting its translation: downregulation of p70S6K1 increases the levels of two proangiogenic factors, HIF1 (hypoxia-inducible factor 1) and VEGF (vascular endothelial growth factor)[79]. MiR-145 also targets the oncogene MYC (v-Myc avian myelocytomatosis viral oncogene homolog), reducing its expression: this explains mR-145-dependent inhibition of cell proliferation *in vivo* and *in vitro*[80]. MiR-21 is the most commonly upregulated miRNA in cancer, including CRC[81]. There are many notable downstream effects of elevated miR-21 levels: its genetic locus at 17q23 is amplified in many solid tumours[82,83]; its expression is stimulated by a variety of cancer-associated phenomena, such as inflammation and hypoxia[84-86]. MiR-21 targets various tumor suppressor genes, such as PDCD4 (programmed cell death 4), CCL20 [chemokine (C-C motif) ligand 20], CDC25A (cell division cycle 25 homolog A), PTEN (phosphatase and tensin homolog), thus promoting cell proliferation, invasion/intravasation/metastasis in CRC[87-90].

In addiction to miR-21, many other miRNAs can be induced in cancer cells under hypoxic conditions[91]. In this group of so called "hypoxamir" there is miR-210, which can mediate the hypoxia-induced metastasis of CRC cells[92]. MiR-210 is frequently up-regulated in CRC tissues, 2D, and 3D cultures[92,93]. Its enforced expression in CRC cells promotes the migration and invasion through the repression of its target VMP1 (vacuole membrane protein 1)[92]. Several investigations reported miR-31 upregulation in CRC[94,95]. Over-expression of miR-31 in CRC cells promotes cell proliferation, invasion, and migration in *in vivo* and *in vitro* models. Likely, its oncogenic functions are due to targeting of SATB2 (*SATB homeobox 2*) mRNA, which is followed by SATB2 mRNA and protein downregulation, and by targeting E2F2 (E2F Transcription Factor 2): in turn, E2F2 controls the expression of survivin and other cell cycle genes, such as CCNA2 (*Cyclin A2*), CDK2 (cyclin-dependent kinase 2), MCM4 (minichromosome maintenance complex component 4), and MYC[96,97]. Notably, members of the E2F family may have a dual function: it has been hypothesized that they may act as oncogenes when they are overexpressed and as tumor suppressors when they are downregulated[96]. MiR-31 also targets RASA1 (RAS p21 GTPase activating protein 1) and inhibits its translation, activating the KRAS signalling pathway[98]. One of the most known oncogenic miRNA clusters in CRC is the miR-17-92 cluster. It consists of six members (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) with oncogenic functions, all upregulated in CRC[99]. MiR-18a and miR-19 promote angiogenesis by targeting TSP-1 (thrombospondin-1) and CTGF (connective tissue growth factor) mRNAs, respectively[100,101]. Interestingly, different miR-17-92 cluster members can modulate cell proliferation in opposite ways. MiR-19a and miR-19b induce proliferation by acting on PTEN, whereas miR-18a has antiproliferative effects due to its target genes that activate proliferation, such as NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) and CDK19 (cyclin-dependent kinase 19)[102]. MiR-17 and miR-20a target E2F1 (E2F transcription factor 1), whereas miR-20a represses E2F2 and E2F3 (E2F transcription factor 3)[103]. Among miR-17-92 cluster members, only miR-92a has antiapoptotic effects: a negative correlation has been described between miR-92a and BCL2L11 (BCL2-like 11), a pro-apoptotic BCL-2 (B-cell CLL/lymphoma *2*) protein family member[101]. Among miR-17-92 cluster members, miR-18a seems to act as a tumor suppressor in CRC: some studies have shown that this miRNA can affect cell proliferation by inhibiting CDC42 (cell division cycle 42)[103], or can promote apoptosis causing hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1) autophagosomal degradation[104]. CDC42 is a small Ras-related GTPase involved in cell cycle progression[105], transendothelial migration through β1 integrin[106], cell motility and cytoskeletal remodelling[107]. The let-7 family is one of the most ancient and conserved group of miRNAs[108]: they act as tumor suppressors in various cancer models, including CRC[109-111]. The let-7 family owes its name to the *lethal-7* gene, identified for the

first time in *C. elegans*, where it is involved in development. Later, the same sequence was detected in the *genome* of *Drosophila melanogaster* and *Homo sapiens*, confirming that mature let-7 is highly conserved across animal species. However, the number of members of this family varies in different species: the let-7 family in humans includes 10 mature miRNAs produced from 13 precursor sequences[112]. Several studies have demonstrated that the let-7 family regulates KRAS expression in CRC and other cancer types[111-114]. KRAS is a small monomeric GTPase, involved in signal transduction of stimuli activating proliferation[115]. KRAS mutations or amplifications are frequently detected in CRC patients[116]: they are considered a key step in colorectal carcinogenesis according to the model proposed by Vogelstein[6]. Moreover, KRAS mutation status is a negative predictive factor of the response to anti-EGFR therapy (*i.e.*, Cetuximab)[117]. It has been reported that let-7b and let-7e were downregulated after Cetuximab treatment in a Cetuximab-resistant CRC cell line, suggesting their potential role in the resistance to anti-EGFR therapy[118]. Several reports debated the predictive utility of a let-7 microRNA-binding-site polymorphism in the 3'-UTR of KRAS for CRC outcome, although the results are conflicting[114,119-121]. Together with other let-7 family members, let-7c modulates cell cycle by targeting KRAS, and is also involved in suppressing metastasis *via* its targets MMP1(matrix metallopeptidase 1) and PBX3 (pre-b-cell leukemia homeobox 3). It has been demonstrated that ectopic expression in Lovo cells or inhibition of let-7c in HT29 cells reduces cell migration and invasion and increases cell motility and invasion, respectively[122]. Overactivation of KRAS signalling could induce the expression of oncogenic miRNAs in CRC. For instance, miR-372 expression is higher in KRAS-mutated CRC samples compared with wild type tumours[123]. miR-372 knockdown decreases cell proliferation and migration and increases apoptosis in CRC cell lines[123]. MiR-372 downregulation results in TXNIP (thioredoxin-interacting protein) overexpression[123]: TXNIP is a tumor suppressor gene involved in apoptosis induction and cell proliferation inhibition[124]. High miR-372 expression is significantly associated with liver metastasis: metastatic CRC samples show higher miR-372 expression than non-metastatic tumours; also, high miR-372 expression is associated with lower 5-year overall survival rate[125]. Similar to miR-372, also miR-720 was found to be more expressed in CRC with mutated KRAS than wild-type KRAS[123,126]. Its overexpression correlates with tumour size, spreading of metastases to distant sites and low 5-year overall survival[126]. MiR-720 knockdown reduces cell proliferation, migration and invasion, and induces apoptosis in CRC cell lines[123,126]. The same miR-720 regulates STARD13 [star-related lipid transfer (START) domain containing 13] expression[126], a GTPase-activating protein (GAP) for Rho and Cdc42. It has been demonstrated that STARD13 knockdown induces upregulation of the antiapoptotic protein BCL2, downregulation of proapoptotic BAX (BCL2-associated X protein), and promotes 3D motility[127]. The miR-200 family is one of the best-known miRNA families in mammals. It consists of five members, which are located at two different loci of chromosome 1 (miR-200a, miR-200b, miR-429) and 12 (miR-200c and miR-141)[128,129]. They are tumor suppressor miRNAs and are significantly involved in inhibition of epithelial-to-mesenchymal transition (EMT), repression of cancer stem cell self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance[128-130]. MiR-200c is particularly interesting in CRC, due to its ability to regulate cell proliferation, invasion, migration, EMT and metastasis. MiR-200c expression is statistically lower in CRC clinical specimens and highly metastatic CRC cell lines. Transfection of CRC cell lines (RKO and SW620) with precursors of miR-200c induced cell proliferation, but reduced invasion and migration. Overexpression of miR-200c in CRC cell lines caused a reduced expression of its target ZEB1/2 (zinc finger E-box binding homeobox 1/2), and resulted in increased E-cadherin and reduced vimentin expression. The associations between miR-200c, its target genes, and EMT markers were validated in primary CRCs and matching liver metastasis tissues[131]. MiR-200c targets SOX2 [SRY (sex determining region Y)-box 2], a pivotal gene required for early development and propagation of undifferentiated embryonic stem cells. Knockdown of miR-200c increased the sphere-forming capacity of CRC cell lines and expression of CRC stem cell markers. MiR-200c suppresses the expression of SOX2, so repressing the activity of the PI3K (phosphoinositide 3-kinase)/AKT (v-Akt murine thymoma viral oncogene homolog 1) pathway[132]. Another tumor suppressor miRNA family controlling EMT in CRC is the miR-34 family. It includes three oncosuppressor members (miR-34a, miR-34b and miR-34c), which are regulated by p53; by promoting mesenchymal-to-epithelial transition (MET) *via* the inhibition of the EMT-inducing transcription factor SNAI1 (snail family zinc finger-*1*), they are involved in metastasis suppression. MiR-34a targets involved in CRC invasion and metastasis include IL6R (interleukin 6 receptor), ZNF281 (zinc finger protein 281), MET (MET proto-oncogene, receptor tyrosine kinase), SNAI1, CTNNB1 (β-catenin) and SNAI2 (snail family zinc finger 1)[133-136]. A common event in CRC carcinogenesis is the inactivation of APC (adenomatous polyposis coli), a negative regulator of Wnt signalling pathway through binding to β-catenin, together with Axin and Glycogen Synthase-3, followed by degradation through ubiquitination. Inactivated APC cannot interact with β-catenin, which accumulates in the cytoplasm and then translocates into the nucleus: here β-catenin binds the TCF/LEF (transcription factor/lymphoid enhancer-binding factor) transcription factors, thus leading to MYC and cyclin D transcription and induction of cell proliferation. An miRNA-based regulation of APC has been proposed: miR-135b binds APC mRNA regulating its expression; miR-135b upregulation in CRC cells

causes reduced APC protein levels, β-catenin accumulation and Wnt pathway activation[137]. Another study proved that miR-135b also affects apoptosis through its targets TGFβR2 (transforming growth factor β receptor 2) and DAPK1 (death-associated protein kinase 1), both frequently downregulated in CRC[138]. Similar to miR-135b, also miR-135a is frequently upregulated in CRC[139,140]. Acting as an oncomiR, miR-135a promotes cell proliferation, motility and invasion of CRC cells. A target of miR-135a is MTSS1 (metastasis suppressor-1), which is downregulated in CRC, promoting malignant phenotypes *in vitro*[141]. Interestingly, the oncogenic effects of miR-135b on the Wnt pathway can be counterbalanced by the tumor suppressive action of miR-320a on the same pathway[142-144]. Overexpression of miR-320a in CRC cell lines reduces cell proliferation, blocking cell cycle in G1. Cell cycle arrest is due to miR-320a and β-catenin mRNA interaction, which results in decreased levels of β-catenin protein, together with those of its transcriptional target genes: MYC, cyclin D, survivin[142]. MiR-320a expression inversely correlates with proliferation and migration of CRC cells and is significantly lower in metastatic compared with non-metastatic samples[143,144]. MiR-320a overexpression suppresses migration and invasion by targeting RAC1 (ras-related C3 botulinum toxin substrate-1). It has also been observed that miR-320a overexpression induces vimentin downregulation and E-cadherin upregulation, suggesting that miR-320a is also involved in EMT regulation[144]. Wnt signalling is also controlled by miR-181a. Its upregulation in CRC cell lines induces cell proliferation by means of its WIF1 target (WNT inhibitory factor-1), which is involved in apoptosis promotion, and PTEN, involved in the AKT signalling pathway[145,146]. Moreover, miR-181a overexpression causes downregulation of the epithelial markers E-cadherin and β-catenin and increased expression of vimentin, suggesting that miR-181a also promotes EMT in CRC[145]. MiR-106a acts as an oncogene in CRC, and its upregulation leads to decreased protein levels of RB1 (retinoblastoma protein-1)[81,147]. Active (unphosphorylated) RB1 binds transcription factors E2F and TFDP1 (transcription factor DP-1) at the promoter of the E2F-regulated genes, which are involved in cell cycle progression. The presence of the RB/TFDP1/E2F complex at the promoter inhibits transcription and recruits chromatin-remodelling complexes, inducing gene silencing and blocking cell cycle progression[148]. MiR-106a binds to the 3'UTR of the RB1 mRNA, inhibiting its translation and inducing cell cycle progression in CRC[147]. By targeting TGFβR2, a tumor suppressor commonly inactivated in CRC, miR-106a is also involved in migration and invasion *in vitro* and *in vivo*[149,150]. Upregulation of miR-155 in CRC has been shown by several studies[151-153]. It has been demonstrated that this oncomiR promotes migration and invasion by targeting the CLDN1 (claudin 1) protein, a component of tight junctions[152]. In turn, CLDN1 is involved in EMT, causing E-cadherin upregulation through ZEB1 downregulation[154]. Adrenaline-induced miR-155 upregulation modulates cell proliferation and chemoresistance in HT29 CRC cell line. Adrenaline increases miR-155 levels *via* NFκB (nuclear factor kappa-light-chain-enhancer in activated B cells), inducing cell proliferation and inhibiting cisplatin-induced apoptosis[153]. It has been recently demonstrated that cyclo-oxygenase 2/prostaglandin-endoperoxide synthase 2 (COX2/PTGS2) and prostaglandin E2 (PGE2), a COX2 metabolite, play an important role in colon cancer progression and are potential targets for prevention and therapeutic strategies[155,156]. COX2 is the inducible isoform of the key enzyme in prostaglandin biosynthesis and it has been associated with several malignancies, including CRC[157,158]. Its upregulation promotes cell proliferation *in vitro* and *in vivo*[159]. MiR-101 acts as a tumor suppressor in CRC patients and cell lines[158,160]. Among miR-101 targets are COX2 and PTGER4 (prostaglandin E receptor 4), a G protein-coupled cell surface receptor involved in PGE2 signal transduction[158,161]. MiR-101 negatively regulates PTGER4: both miR-101 overexpression and PTGER4 silencing reduce motility and colony formation *in vitro*[161]. MiR-101 overexpression also promotes cell adhesion and inhibits colonosphere formation, cell growth, invasiveness and survival in hypoxic conditions[160]. MiR-101 repression and Wnt signalling pathway activation show a strong association: miR-101 overexpression reduces β-catenin accumulation in the nucleus and transcriptional activity, leading to increased E-cadherin and decreased ZEB1 mRNA levels; this suggests miR-101 involvement in EMT regulation[160]. MiR-638 downregulation promotes EMT through its target SOX2[162]: it has been demonstrated that SOX2 overexpression induces dedifferentiation and EMT[163]. MiR-638 expression is reduced in CRC tissues and cell lines: its downregulation inversely correlates with tumour progression and predicts poor survival[162,164]. MiR-638 ectopic expression in CRC cell lines results in a reduction of migration, invasion and cell proliferation: this is due to overexpression of its target TSPAN1 (tetraspanin 1)[161,164], which is involved in CRC cell cycle and invasion regulation[165]. MiR-1 levels are reduced in CRC compared with normal tissues: this significantly correlates with MET gene overexpression. Moreover, miR-1 ectopic expression in CRC cell lines impairs MET-induced cell viability, migration and invasion[166]. MiR-23b acts as a tumor suppressor in CRC cell lines by performing a pleiotropic modulation of different cancer-related biological processes. Its ectopic expression strongly inhibits migration and invasion *in vitro* and primary tumour growth and metastasis *in vivo*. MiR-23b overexpression reduces cell resistance to *anoikis*, programmed cell death induced by detachment of anchorage-dependent cells from the extracellular matrix. MiR-23b may also promote mesenchymal-to-epithelial transition: it has been observed that its expression induces E-cadherin upregulation and vimentin reduction. These tumor suppressive effects are

due to miR-23b pro-metastatic targets, FZD7 (frizzled class 7 receptor), MAP3K1 (MEKK1, mitogen-activated protein kinase kinase 1, E3 ubiquitin protein ligase), PAK2 [p21 protein (Cdc42/Rac)-activated kinase 2], TGFβR2, RRAS2 [related RAS viral (r-Ras) oncogene homolog 2], and uPA (plasminogen activator, urokinase). Furthermore, miR-23b overexpression inhibits angiogenesis by indirectly suppressing VEGF through FZD7 and MAP3K1[167]. Several papers reported the downregulation of miR-126 in CRC tissues and cell lines[168-170]. Methylation studies suggest that miR-126 is epigenetically silenced by CpG methylation of the promoter of its host gene EGFL7 (EGF-like-domain multiple 7)[171]. Through its target PIK3R2 (phosphoinositide-3-kinase, regulatory subunit 2), a regulatory subunit involved in the PI3K signalling pathway, miR-126 ectopic expression results in cell proliferation impairment[168]. MiR-126 also binds the mRNAs encoding IRS1 (insulin receptor substrate 1) and CXCR4 [chemokine (C-X-C motif) receptor 4], thus regulating AKT and ERK1/2 (mitogen-activated protein kinase 3/1) activation. MiR-126 inhibits cell proliferation, inducing G0/G1 arrest, migration and invasion in CRC cell lines[168-171]. It has been shown that miR-126 ectopic expression in CRC cell lines impairs VEGF secretion in culture medium, suggesting that miR-126 affects angiogenesis[171]. Similarly, DNA hypermethylation of CpG islands seems to cause miR-148a downregulation in various cancers. In CRC cell lines, miR-148a upregulation promotes apoptosis through BCL2 inhibition[172]. Moreover, miR-148a downregulation in CRC correlates with increased tumour size[173]. All data reported in this paragraph (*i.e.*, CRC related miRNAs, their functions and targets) are summarized in Table 1.

The impact of lncRNAs on CRC pathobiology

Over the last few years, several papers have reported on the involvement of lncRNAs in CRC genesis and progression through a number of molecular mechanisms. LncRNAs impact on critical CRC signalling pathways by acting both as oncogenes and tumor suppressors through interactions with other regulatory molecules, such as DNA, RNA, and proteins. Although several lncRNAs have been reported to be dysregulated in CRC, which suggests their potential diagnostic/prognostic power (see paragraph "Clinicopathological significance of lncRNAs in CRC"), their molecular mechanisms of action in CRC biology were elucidated only for few of them. One of the most known cancer-related lncRNAs is Metastasis-Associated Lung Adenocarcinoma transcript 1 (MALAT1), located on chromosome 11q13.1 and 8000 nt long[174]. MALAT1 is highly expressed in metastases of various tumours, such as non-small cell lung cancer, hepatocellular carcinoma, and endometrial stromal sarcoma[174-176]. Several intracellular functions of MALAT1 have been proposed. It may have an important role in pre-mRNA metabolism: it is indeed associated with SC35 splicing domains within the nucleus[177]. It is concentrated in nucleoli as a "riboregulator", controlling expression of its target genes[178]. Moreover, MALAT1 is involved in the regulation of tumor suppressor proteins [*e.g.*, PTB-associated splicing factor (PSF)][179], and has also been found to regulate the activity of E2F1, a pivotal transcription factor for cell cycle progression[180]. *In vitro* silencing of MALAT1 has been shown to affect bladder cancer cell migration. MALAT1 acts as a negative regulator of EMT-associated ZEB1, ZEB2 and SNAI2, and positive regulator of E-cadherin[181]. Aberrant mitosis, with a large fraction of cells accumulating at the G2/M boundary and increased cell death, result from MALAT1 depletion[182]. Recently, a 3' end processing mechanism for MALAT1 has been identified: the primary transcription product of the MALAT1 locus is a 6.7 kb nuclear-retained lncRNA and a cytoplasmic 61-nt tRNA-like ncRNA, known as mascRNA (MALAT1-associated small cytoplasmic RNA)[67]. When the MALAT1 RNA fragment containing mascRNA was overexpressed in CRC cells, cell proliferation and invasion were induced. Point mutations of MALAT1 were detected in CRC cell lines and tissues[183]. BRAF-activated non-protein coding RNA (BANCR) seems to be closely associated with ^{V600E}BRAF, one of the most frequent mutation types of the BRAF (B-Raf proto-oncogene, serine/threonine kinase) gene in several tumours, including CRC[184]. BANCR is frequently overexpressed in CRC tissues: this overexpression significantly correlates with lymph node metastasis and tumour stage[185]. Enforced expression of BANCR increases cell migration of CRC cell lines, whereas its knockdown inhibits it. BANCR induces EMT by affecting the expression of epithelial and mesenchymal markers through a MEK (mitogen-activated protein kinase kinase 7)/ERK dependent mechanism, thus contributing to CRC migration[185]. Genome wide association studies (GWAS) have identified a set of risk *loci*, which are linked to susceptibility for different diseases (including CRC) on human chromosome 8q24[186]. Interestingly, this region (about 2 Mb) is within a large protein-coding-gene desert, but several non-coding genes map on it (*i.e.*, CCAT1, CCAT2, PCAT2, and PRNCR1)[187]. The rs6983267 SNP (single nucleotide polymorphism), mapping to the chromosomal region 8q24.21, has been strongly associated with an increased risk of CRC[188]. The genomic region spanning rs6983267 was found to contain DNA enhancer elements, and the allelic variants confer different binding affinity to TCF7L2 [transcription factor 7-like 2 (T-cell specific, HMG-box)], a transcription factor that has a central role in the transcriptional activation of Wnt target genes[189,190]. The SNP status of lncRNA colon cancer associated transcript 2 (CCAT2), which encompasses rs6983267, affects CCAT2 expression: the risk allele G produces more CCAT2 transcripts in CRC[191]. CCAT2 interacts with TCF7L2 and upregulates MYC, miR-17-5p and miR-20a; it also overactivates Wnt signalling[191]. Interestingly, CCAT2 is itself a Wnt downstream target, which suggests the existence of a positive feedback loop[191]. The long

Table 1 Functions of microRNAs deregulated in colorectal cancer

microRNA	Oncogene/tumor suppressor	Process in CRC	Targets	PMID
Let-7	Tumor suppressor	Cell proliferation, migration, invasion, metastasis	KRAS, MMP11, PBX3	23167843, 21984339, 16651716
miR-1	Tumor suppressor	Cell proliferation, invasion, migration	MET	22179665
miR-16	Tumor suppressor	Cell proliferation	PTGS2	22049153
miR-17-92 cluster	Oncogene	Angiogenesis, proliferation, metastasis	TSP-1, CTGF, PTEN, BCL2L11, E2F1, E2F2, E2F3, TGFBR2, CDKN1A, BIM	19460962, 16878133, 22308110, 24212931, 21883694
miR-18a	Tumor suppressor	Cell proliferation, migration	CDC42, HNRNPA1	25379703, 24166503
miR-21	Oncogene	Cell proliferation, migration, invasion, metastasis, stemness	PDCD4, CCL20, Cdc25A, TGFBR2, PTEN, RHOB, RASA1	22677902, 17968323, 22099878, 19826040, 22120473, 23788041, 23544170, 23174819, 22072622, 21872591, 25663768
miR-23b	Tumor suppressor	Cell migration, invasion, angiogenesis	FZD7, MEKK1, PAK2, TGFBR2, RRAS2, PLAU, VEGF	22109528
miR-31	Oncogene	Cell proliferation, invasion, migration	CDKN2B, RASA1	21062447, 25202407, 23322774
miR-34	Tumor suppressor	Migration, invasion, metastasis, EMT	IL6R, ZNF281, MET, SNAIL, CTNNB1, SLUG, ZEB1	24642471, 24185900, 23243217, 22134354
miR-101	Tumor suppressor	Cell proliferation, motility, invasion	EP4, PTGS2	22353936, 19133256
miR-103/miR-107	Oncogene	Invasion, migration, metastasis	DAPK, KLF4	22593189
miR-106a	Oncogene	Cell proliferation, migration, invasion, metastasis	RB1, TGFBR2	23178825, 22912877
miR-126	Tumor suppressor	Cell proliferation, migration, invasion, metastasis, angiogenesis	PIK3R2, IRS1, CXCR4, VEGF	18663744, 24312276, 24189753, 24653631, 23900443
miR-135a	Oncogene	Cell proliferation, migration, invasion	MTSS1	23017832
miR-135b	Oncogene	Cell proliferation, invasion	APC, TGFBR2, DAPK1	18632633, 24735923
miR-143	Tumor suppressor	Cell proliferation, metastasis	KRAS, ERK5, MACC1, HK2, IGF1R, DNMT3A	19137007, 16969504, 22533346, 22691140, 23574723, 19638978
miR-145	Tumor suppressor	Cell proliferation, angiogenesis	MYC, CDK6, E2F1, CCND2, p70S6K1, PAK4	21278451, 15944709, 19843336, 21917858, 22766504
miR-148a	Tumor suppressor	Cell proliferation	BCL2	21455217
miR-155	Oncogene	Cell proliferation, migration, invasion, chemoresistance	CLDN1	23588589, 23036199
miR-181a	Oncogene	Cell proliferation, invasion, metastasis, EMT	PTEN, WIF1	24685694, 24755295
miR-200c	Tumor suppressor	Cell proliferation, invasion, migration, EMT, metastasis	ZEB1, ETS1, FLT1, CDH1, VIM	22735571, 22407310
miR-210	Oncogene	Cell migration, invasion	VMP1	24632577
miR-320a	Tumor suppressor	Cell proliferation, migration, invasion, metastasis, EMT	CTNNB1, RAC1, NRP1	22459450, 24265291, 22134529
miR-372	Oncogene	Cell proliferation	TXNIP, LATS2	22660396, 22456107
miR-638	Tumor suppressor	Cell invasion, migration	SOX2, TSPAN1	24885288, 25301729
miR-720	Oncogene	Cell proliferation, invasion, migration	STARD13	25286763, 22660396

For all miRNAs are reported: (1) their function in CRC (oncogene, or tumor suppressor); (2) the biological processes they are involved in; (3) their mRNA validated targets; (4) bibliographic references reported as PubMed ID (PMID).

isoform of colon cancer associated transcript 1 (CCAT1-L) is upregulated and positively related to tumour stage and progression in CRC[192]. CCAT1-L is located in the nucleus, while the short isoform (CCAT1-S) is found in the cytoplasm. CCAT1-L can interact with a transcriptional enhancer of MYC (MYC-335) by chromatin looping, which in turn interacts with the MYC-promoter[193]. Knockdown of CCAT1-L leads to a decreased level of MYC mRNA, strongly suggesting that this lncRNA may regulate MYC expression in *cis*[193]. Another lncRNA locus mapping at 8q24 is Plasmacytoma Variant Translocation 1 (PVT1): PVT1 is located downstream of MYC on chromosome 8q24 and produces a wide variety of spliced RNAs, such as a cluster of six miRNAs (*i.e.*, miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208)[194]. PVT1 is upregulated in CRC because of a copy number amplification of chromosome 8q24[195]. Knockdown of PVT1 in CRC cells leads to a significant reduction of proliferation and invasion through activation of TGF-β and apoptotic signalling. Increased PVT1 expression is required for high MYC protein levels in 8q24-amplified CRC cells. PVT1 and MYC protein expression correlate in primary tumours, while ablation of PVT1 from MYC-driven colon cancer line HCT116 diminished its tumorigenic potential[195]. Surprisingly, PVT1 is also a p53-inducible target gene: p53 binds and activates a canonical response element within the vicinity of miR-1204, and induces the endogenous PVT1 transcripts and consequent upregulation of miR-1204[196]. Ectopic expression of miR-1204 leads to increased p53 levels and causes cell death in a partially

p53-dependent manner[196]. The most intensively studied lncRNA in different neoplastic diseases is the Hox transcript antisense intergenic RNA (HOTAIR). It is located within the Homeobox C (HOXC) gene cluster on chromosome 12 and is coexpressed with HOXC genes[197]. HOTAIR interacts with PRC2; it functions as a scaffold to assemble PRC2 on the HOXD gene cluster, inducing the trimethylation of histone H3 lysine-27 (H3K27me3) of the HOXD locus[198]. By silencing multiple metastasis suppressor genes (such as HOXD10, PGR, and the protocadherin gene family), HOTAIR epigenetically regulates HOXD expression and promotes metastasis in breast cancer[199]. Expression analysis on CRC reveals a close correlation between HOTAIR expression and members of the PRC2 complex (*i.e.*, SUZ12, EZH2, and H3K27me3): this suggests that HOTAIR expression is associated with epigenetic functions of PRC2, which is involved in maintaining the mesenchymal and undifferentiated status in CRC cells[200]. Similarly to HOTAIR, also the lncRNA CRNDE (colorectal neoplasia differentially expressed) has been shown to be physically and functionally associated to PRC2[201]. Khalil *et al*[201] showed an overlap in the lists of genes affected by knockdown of CRNDE and PRC2. These data would suggest an involvement of CRNDE in the epigenetic remodelling of chromatin, and specifically in the downregulation of gene expression *via* targeted histone methylation by the PRC2 complex. The CRNDE promoter is bound by some pluripotency-related transcription factors [*i.e.*, MYC, MYCN (v-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog)] and CRNDE knockdown decreases the expression of several pluripotency markers (*i.e.*, SOX2, KLF4, NANOG, and OCT4)[202]. CRNDE expression appears highest in the early stages of mammalian development and progressively decreases thereafter. It is required for the maintenance of pluripotency in mouse embryonic stem cells and is potentially involved in tumorigenesis[202]. The tumor suppressor candidate 7 (TUSC7) is a p53-regulated tumor suppressor, which reduces tumour cell growth both *in vitro* as well as *in vivo* in CRC[203]. Specifically, its fourth exon (containing two miR-211 binding sites) is responsible for inhibition of tumour cell growth. *In vivo* studies confirmed that TUSC7 can bind to miR-211 inducing its down-regulation[203]. It has been shown that miR-211 promotes cell growth in CRC cell lines. Accordingly, TUSC7 works as an endogenous miRNA sponge. Interestingly, TUSC7 is a target of miR-211, showing the existence of a reciprocal negative feedback loop between TUSC7 and miR-211[203]. H19 is a paternally imprinted, maternally expressed, oncofetal gene[204]. It is highly expressed from the early stages of embryogenesis to fetal life in many organs, but is nearly completely downregulated postnatally[205]. H19 is upregulated in many cancers, including CRC[206], and acts as the primary miRNA precursor of miR-675 in mammals[207]. RB1 mRNA is a direct target of miR-675: in fact, knockdown of miR-675 increases RB1 expression and at the same time decreases cell growth in CRC. On the contrary, miR-675 gain-of-function causes RB1 downregulation and enhances tumour cell growth. Both TGF-β and hypoxia concomitantly induce H19 and miR-675, together with induction of EMT markers and suppression of E-cadherin protein expression[207]. Interestingly, H19 harbours both canonical and non-canonical binding sites for the let-7 miRNA family, whose critical tumor suppressive role in the development of CRC has already been discussed above. H19 is able to downmodulate let-7 availability by acting as a molecular sponge[208]. Recently, the role of another lncRNA, functionally linked to EMT, has been characterized in CRC[209]. N-BLR is an lncRNA involved in the apoptotic pathway: its inhibition leads to downregulation of XIAP (X-linked inhibitor of apoptosis protein) and subsequent increase of cell death. N-BLR also promotes invasion and migration by modulating vimentin and E-cadherin expression. Intriguingly, N-BLR seems to be regulated by members of the miR-200 family (*i.e.*, miR-141, and miR-200c). As previously mentioned, the miR-200 family has been strongly linked to EMT: indeed, its members target the ZEB1/ZEB2 transcription factors that are repressors of E-cadherin expression. According to the model proposed by Rigoutsos *et al*[209], the increase of N-BLR expression in CRC samples would attract the available endogenous miR-141/miR-200c and relieve their targeting ZEB1, thereby upregulating its levels. This would lead to a subsequent decrease of E-cadherin; it also would confer a mesenchymal phenotype to the cells, which is correlated with increased invasiveness and migratory potential of CRC cells. All data reported in this paragraph (*i.e.*, CRC related lncRNAs, their functions and targets) are summarized in Table 2.

Circular RNAs: New players in cancer regulation?

Recently, it has been discovered that hundreds of human genes are also expressed in a circular RNA isoform[210]. Initially, these circRNAs were thought to be rare RNA species representing just transcriptional noise. High-throughput sequencing of the RNase R treated, ribosomal-depleted fractions of RNAs showed a ubiquitous expression of circRNAs in human and mouse cells[211]. CircRNAs represent a class of little known post-transcriptional regulators, which compete with other RNAs for binding to miRNAs and RNA binding proteins (RBPs): they may have a role in modulating the local concentration of RBPs and RNAs, as part of the competing endogenous RNA network[211]. Moreover, in contrast with classical ceRNAs, circRNAs have no accessible termini, which makes them resistant to miRNA-mediated RNA degradation or other exonucleolytic activities. ciRS-7 [also termed CDR1as (cerebellar degeneration-related protein 1 antisense)], one of the most studied circRNAs, is a circular miR-7 inhibitor/sponge that binds miR-7, resulting in reduced

Table 2 Functions of long non-coding RNAs deregulated in colorectal cancer

lncRNA	Oncogene/tumor suppressor	Biological process	Target	PMID
AK123657	Tumor suppressor	Cell proliferation, invasion		24809982
BANCR	Oncogene	Cell migration, EMT		25013510
BX648207	Tumor suppressor	Cell proliferation, invasion		24809982
BX649059	Tumor suppressor	Cell proliferation, invasion		24809982
CCAT1-L	Oncogene	MYC expression regulation	MYC-335	24662484
CCAT2	Oncogene	WNT signalling pathway activation	TCF7L2, MYC, miR-17-5p, miR-20a	23796952
CRNDE	Oncogene	Epigenetic remodelling of chromatin		19571010
H19	Oncogene	miR-675 precursor, cell proliferation, EMT, miRNA sponge	let-7	17237358, 19926638
HOTAIR	Oncogene	HOXD expression regulation, metastasis	HOXD10, PGR	24075995, 20393566
MALAT1	Oncogene	pre-mRNA metabolism, target gene expression regulator, tumor suppressor protein regulation, cell cycle progression, cell migration, MET	PSF, E2F1	17270048, 16878148, 18067128, 22078878, 22722759
mascRNA	Oncogene	Cell proliferation, invasion		21503572
N-BLR	Oncogene	Apoptosis, cell migration, invasion		http://dx.doi.org/10.1101/004796
PVT1	Oncogene	miRNAs precursor, cell proliferation, invasion		18194563, 25043044
TUSC7	Tumor suppressor	Cell proliferation, miRNA sponge	miR-211	23558749

For all lncRNAs are reported: (1) their function in CRC (oncogene, and tumor suppressor); (2) the biological processes they are involved in; (3) their validated targets; (4) bibliographic references reported as PubMed ID (PMID). LncRNA: Long non-coding RNA.

miR-7 activity and increased levels of miR-7 targets[212]. As miR-7 negatively controls the expression of several oncogenes, impairing miR-7 activity would have an important impact on the cell phenotype. Recently, Bachmayr-Heyda *et al*[213] found a global reduction of circRNA abundance in CRC cell lines and CRC tissues compared with normal tissues and detected a negative correlation between circRNA expression and proliferation. The authors explained these findings by hypothesizing that the back-splice machinery, responsible for RNA circularization, is dysfunctional in tumoral cells; otherwise, downregulation of circRNAs could be due to increased degradation by oncomiRNAs which are deregulated in CRC[213].

NON-CODING RNAS AS DIAGNOSTIC AND PREDICTIVE TOOLS

miRNAs as diagnostic and prognostic CRC biomarkers

As previously reported, there is overwhelming evidence indicating that post-transcriptional and translational controls, mediated by various miRNAs, exert critical pleiotropic actions on different features of CRC evolution. Based on these premises, tremendous effort was made towards the discovery and characterization of miRNAs as predictive and prognostic biomarkers in CRC. Unsurprisingly, most miRNAs involved in CRC regulation also exhibit potential predictive/prognostic properties (Table 3). MiR-31 is the most frequently mentioned in miRNA-based biomarker discovery studies for CRC patients. MiR-31 is upregulated in colon cancer tissues, compared with adjacent non-neoplastic normal tissues, across all clinical stages; its expression correlates with clinical stages[94,97,214]. It was observed that upregulation of miR-31 in tumour samples is positively associated with advanced tumour-node-metastasis (TNM) stage, presence of lymph node metastasis, and distant metastases[94,97,214]; furthermore, high expression of miR-31 correlates with patients' short survival[97]. On the other hand, Slaby *et al*[95] detected upregulation of miR-31 in CRC tissues, but surprisingly found no association with tumour stage. They reported a low expression of miR-31 mainly in poorly differentiated tumours[95]. MiR-21 has been frequently reported as being involved in many neoplasias, including CRC. It has also been demonstrated that it possesses diagnostic and prognostic power. High miR-21 levels correlate with short disease-free survival[215], clinical stage and distant metastases[95]. Analyzing colon and rectal cancer tissues separately, overexpression of miR-21 was an independent prognostic factor of unfavourable recurrence-free survival only for T3-4a colon cancer patients[216]. MiR-21 levels increase, while miR-143 and miR-145 expression decrease, going from well (G1) to poorly (G3) differentiated tumours. Decreased miR-143 and miR-145 expression is also preferentially associated with increased tumour size and localization in the proximal colon[95]. Vickers *et al*[217] reported that miR-21, miR-135a and miR-335 were upregulated in CRC compared with normal adjacent tissues, in particular in metastatic primary tumours, whereas miR-206 levels inversely correlated with CRC progression. Moreover, let-7a showed elevated expression in metastatic CRC compared with normal mucosa or non-metastatic disease, but only in KRAS-mutated tumours. This prognostic signature of miR-21, miR-135a, miR-206, miR-335, and let-7a, used to detect the presence of metastases, had a specificity of 87% and sensitivity of 76%: these data suggest their application as a prognostic tool in CRC[217]. Díaz *et al*[218]

Table 3 Diagnostic and prognostic cellular microRNAs in colorectal cancer

Clinical feature	Oncogenes	Tumor suppressor genes	PMID
Fluorouracil based therapy positive response	let-7g, miR-181b, miR-26a-1 (SNP)		18172508, 20585341
Metastasis	miR-21, miR-372, miR-720, miR-181a, miR-135a, miR-335	miR-126, miR-34a, miR-27a (SNP)	22120473, 24653631, 22456107, 25286763, 23243217, 24755295, 25078482
Poor fluorouracil based therapeutic prognosis	miR-21		18230780
Progression		miR-100 (SNP)	20585341
Survival	miR-21, miR-17, miR-181a, miR-181b, miR-372, miR-720, miR-106a, miR-20a, miR-203, miR-423 (SNP), miR-196a-2 (SNP)	miR-200c, miR-320, miR-498, miR-608 (SNP), miR-219-1 (SNP)	18230780, 18079988, 22065543, 24098024, 18676867, 22456107, 25286763, 22028396, 22661538, 22161766
Tumour size	miR-720	miR-143, miR-145	18196926, 25286763
Undifferentiated phenotype	miR-21	miR-143, miR-145	18196926

Cancer-related phenotypes that may be predicted by expression analysis of oncomiRNAs and tumor suppressor miRNAs. miRNAs: MicroRNAs.

showed an association between downregulation of miR-126 and age under 50 on CRC diagnosis. miR-126 inversely correlated with metastasis and its expression levels were significantly lower in metastatic CRC than in localized tumours[219]. The same authors also observed a correlation between high miR-106a expression and 5-year disease-free survival and overall survival[218]. Recurrence-free survival of patients with stage Ⅱ CRC was also independently associated with high expression of miR-320 and miR-498[220]. MiR-181a is upregulated in CRC tissues compared with normal tissues and in liver-metastatic CRC: this suggests its correlation with liver metastasis and also with poor overall survival in EGFR-targeted therapy[145,221]. On the other hand, low miR-181b and let-7g expression correlates with a positive response to 5-fluorouracil-based antimetabolite S-1[222]. Overexpression of miR-15b, miR-181b, miR-191 and miR-200c in CRC, compared to normal colorectal tissues, was reported by Xi *et al*[223]. Kaplan-Meier survival analysis showed that patients with higher miR-200c expression had shorter survival time compared with patients with lower expression[223]. MiR-372 and miR-720 (both controlled by the KRAS pathway) showed high expression levels, which are significantly associated with CRC tumour size and distant metastases. Metastatic CRC samples showed higher miR-372 and miR-720 expression compared with the non-metastatic samples; moreover, their upregulation was found to be associated with lower 5-year overall survival[125,126]. The presence of metastases in CRC patients is also associated with reduced miR-34a expression, caused by high CpG methylation of miR-34a and miR-34b/c promoters[135]. Expression levels of the oncogenic miR-17-92 cluster and two of its paralogs (miR-106a and miR-106b) are significantly elevated in CRC. Although the authors observed no significant association between deregulation of these miRNAs and clinico-pathological features of patients, high levels of miR-17 were related to reduced overall survival of CRC patients[224]. A promising non invasive approach for CRC screening is to assay stools for molecular biomarkers that mirror the molecular alterations associated with cancer: colon cancer tissues consistently shed cancer cells into stools; accordingly, it would be an ideal substrate to detect specific CRC biomarkers. Several papers showed quantitative changes in the expression of some miRNAs in stools of CRC patients with respect to normal controls. Ahmed *et al*[225,226], reported 12 upregulated miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR-214) and 8 downregulated miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) in the stools of CRC patients; and these alterations were more pronounced in later carcinoma stages. MiR-21 and -106a upregulation in stools of adenoma and CRC patients was also reported in different papers[227,228]. Other studies on CRC stools suggested miR-18a, miR-31, miR-135b, and miR-221 as potential biomarkers of adenoma and carcinoma[229,230].

Pathological and clinical roles of lncRNAs in CRC

Several high-throughput profiling and reverse transcription polymerase chain reaction (RT-PCR) studies were published in the last few years that showed the potential diagnostic and prognostic power of lncRNAs in CRC (Table 4). By searching through previously published gene expression microarray data, Hu *et al*[231] analyzed lncRNA profiles of large cohorts of CRC patients and identified a prognostic six-lncRNA signature. These six lncRNAs (*i.e.*, AK024680, AK026784, AK123657, BX648207, BX649059, and CR622106) significantly correlated with disease-free survival: this signature was able to classify CRC patients into two subgroups: high-risk (shortened survival) and low risk (prolonged overall survival). Moreover, functional experiments demonstrated that repression of lncRNAs AK123657, BX648207 and BX649059 in CRC cell lines increased cell proliferation and invasion[231]. One of the most upregulated lncRNAs associated with CRC is CRNDE[232]. CRNDE exists in different splice variants that may have diagnostic usefulness: the most diagnostically

Table 4 Diagnostic and prognostic cellular long non-coding RNAs in colorectal cancer

Clinical feature	Oncogenes	Tumor suppressor genes	PMID
Disease-free survival	AK026784, AK024680, MALAT1	AK123657, CR622106, BX649059, BX648207, TUSC7, RPL34-AS1	24809982, 25031737, 23680400, 24908062
Distant metastasis	91H, PCAT1, CCAT1	TUSC7, RPL34-AS1	25058480, 23640607, 23594791, 23680400, 24908062
Liver metastasis	HOTAIR, HULC	ncRAN	21862635, 24519959, 19445043
Lymph node metastasis	PVT-1, AK021444, ENST00000425785, AK307796	ENST00000465846	24196785, 25009386
Metastasis risk	MALAT1		25031737
Overall survival	MALAT1, PVT-1, PCAT1	ncRAN, uc.73	25031737, 24196785, 24519959, 23640607, 22328099
Plasma diagnostic marker	CRNDE		22393467
Poor prognosis	HOTAIR, 91H, PVT-1		21862635, 25058480, 24196785
Tumour risk	PRNCR1 (SNP)		24330491
Tumour size	HOTAIR, PRNCR1 (SNP)	TUSC7	21862635, 23680400, 24330491
Tumour stage	CCAT1	lincRNA-p21, TUSC7, RPL34-AS1	24012455, 23594791, 23680400, 24908062
Undifferentiated phenotype	HOTAIR, PRNCR1 (SNP)	ncRAN	21862635, 24519959, 24330491
Venous invasion	PVT-1	lincRNA-p21	24196785, 24012455

Cancer-related phenotypes that may be predicted by expression analysis of oncogenic and tumor suppressive long non-coding RNAs.

relevant isoform CRNDE-h showed a sensitivity of 95% and a specificity of 96% for distinguishing adenomas from normal tissues and a sensitivity of 80% and a specificity of 96% for carcinoma *vs* normal tissues[233]. High expression levels of HOTAIR in CRC patients were closely related to poor prognosis[200]. Specifically, Kogo *et al*[200] divided 100 patients with CRC into two groups: a high HOTAIR expression group and a low expression group: their data show that CRC patients with the highest HOTAIR expression exhibit less differentiated histology, greater tumour size, and higher propensity to liver metastasis than CRC patients with low HOTAIR expression. Similar results were obtained for MALAT1 by Zheng *et al*[234], who statistically associated expression of MALAT1 to clinico-pathological parameters, disease-free survival, and overall survival. Stage Ⅱ/Ⅲ CRC patients with higher expression of MALAT1 showed a significantly higher risk of metastasis after radical surgery and significantly poorer overall survival[234]. 91H (also named LINC01219), an antisense RNA of lncRNA H19, is overexpressed in CRC tumour tissues with respect to adjacent normal tissues. Clinico-pathological factors were compared between CRC patients with high and low expression of 91H. Statistical analysis showed that high expression of 91H was significantly correlated with distant metastases and poorer prognosis[235]. LncRNA PVT1 is a precursor of a number of spliced ncRNAs and of six miRNAs. The biomolecular functions of PVT1 remain elusive, even though several studies were performed to determine them[236,237]. Takahashi *et al*[238] investigated the clinical significance of PVT1 expression on 164 CRC patients, showing that high PVT1 expression was positively related to the size of lymph node metastasis, venous invasion and poor prognosis. Furthermore, univariate and multivariate analyses showed that PVT1 expression was an independent risk factor for overall survival of CRC patients. NcRAN (non-coding RNA expressed in aggressive neuroblastoma) is an lncRNA whose expression is highly upregulated in neuroblastoma patients with poor prognosis; it could also have an important pathogenetic role in human bladder cancer[239,240]. Qi *et al*[241] demonstrated that ncRAN expression is significantly reduced in CRC tumour tissues and cell lines, compared with adjacent normal tissues and a normal intestinal mucous cell line. Downregulation of ncRAN was more evident in poorly differentiated or undifferentiated tumours and in CRC with liver metastases. Kaplan-Meier survival analysis showed that CRC patients with lower ncRAN expression had a worse overall survival. Overexpression of PCAT1 (prostate cancer associated transcript 1) was associated with distant metastases and poorer overall survival in CRC patients: in few cases, this was explained by gene copy number variation of the PCAT1 locus[242]. Long intergenic noncoding RNA-p21 (lincRNA-p21) is transcriptionally induced by p53 in CRC cell lines, following apoptosis induction by nutlin-3; it works as a repressor in p53-dependent transcriptional responses. Its expression levels were lower in CRC tumour tissues, when compared with adjacent normal tissues from the same patient. Expression of lincRNA-p21 was higher in rectum cancers with respect to colon cancers. Its overexpression was significantly correlated to higher primary tumour (pT) and vascular invasion[243]. Alaiyan *et al*[244] screened the expression of CCAT1 in normal colon tissues and in various stages of CRC development (*i.e.*, adenoma, invasive carcinoma, lymph nodes metastases, and distant metastases). By using quantitative RT-PCR (qRT-PCR) and *in situ* hybridization (ISH), they found: (1) increased CCAT1 expression in colon adenocarcinoma compared with normal tissues; and (2) heterogeneous upregulation across the colon adenoma-carcinoma sequence. CCAT1 upregulation was evident in premalignant conditions and through all disease stages, including advanced metastatic disease:

these data suggest its potential role both in transformation and in metastasis. Transcribed ultra-conserved regions are a recently discovered class of non-coding RNAs with a high degree of evolutionary conservation: this strongly suggests a critical role in the physiology of mammals and other vertebrates. T-UCRs mainly work as antisense molecules for protein-coding genes and miRNAs; their dysregulation has been reported for different types of tumours[245,246]. Sana *et al*[247] analyzed the expression in CRC tissues and adjacent normal tissues of t-UCR uc.43, uc.73, uc.134, uc.230, uc.339, uc.388, uc.399, which previously had been found to be associated with CRC. Among these t-UCRs, only uc.73 and uc.388 were significantly downregulated in CRC tissues; uc.73 showed a positive correlation with overall survival of CRC patients. The tumor suppressor lncRNA TUSC7 (LOC285194) is depleted in osteosarcoma, causing abnormal proliferation of osteoblasts; its deletions were associated with poor survival of osteosarcoma patients[248]. Qi *et al*[249] found that expression levels of TUSC7 significantly decreased also in CRC samples. In addition, this downregulation was correlated with larger tumour size, higher tumour stage, more distant metastases, and poor disease-free survival[249]. By microarray profiling of CRC samples and adjacent normal tissues from non-metastatic and metastatic patients, six aberrantly expressed lncRNAs (*i.e.*, AK097793, ENST00000423943, ENST00000393516, ENST00000428029, RP11-462C24.1, and uc002wvk.2) were identified[250]. Among these, RP11-462C24.1 (also called RPL34-AS1), whose functions are still unknown, exhibited interesting prognostic properties: its expression level decreased as the malignant degree of CRC increased. Furthermore, downregulation of RP11-462C24.1 significantly correlated with more distant metastases and a poor disease-free survival[250]. Some studies on the diagnostic and prognostic power of lncRNAs in CRC focused on their expression in metastatic sites rather than CRC tissues. LncRNA expression profiling in metastatic lymph nodes (MLN), normal lymph nodes (NLN), and tumour tissues from CRC patients showed that 14 lncRNAs were specifically upregulated (*e.g.*, AK021444, ENST00000425785, and AK307796) and 5 lncRNAs were specifically downregulated (*e.g.*, ENST00000465846) in the MLN group compared with the NLN group and tumour tissue group[251]. These data suggest that specific lncRNA dysregulation in MLN may have an important role in facilitating the occurrence of lymph node metastasis in CRC. However, the molecular functions of these lncRNAs are still unknown. The lncRNA highly upregulated in liver cancer (HULC) is usually upregulated in hepatocellular carcinoma and may perturb the circadian rhythm of hepatoma cells[252,253]. HULC is neither expressed in CRC tissues nor in normal colon mucosa; however, it was found to be significantly expressed in CRC with metastases in the liver, but not in lymph nodes[254]. These data suggest that HULC could play a role in the metastatic process of CRC tumours in the liver.

Non-coding RNAs with predictive power in CRC therapy response

One of the most challenging tasks in cancer treatment is to identify patient subpopulations who could benefit from chemotherapy and avoid over-treatment of chemorefractory patients. Discovery of predictive biomarkers would provide information on the likelihood of response to a given therapeutic agent and help to optimize therapy decisions. Much effort was made to find feasible predictive biomarkers in CRC, however, except for KRAS mutations, no clinical study provided markers which have entered into the clinical management of CRC. Recent studies revealed that expression levels of certain miRNAs could be associated with specific therapeutic outcomes in CRC, suggesting that miRNAs may be considered potential predictive biomarkers. Some *in vitro* studies on CRC cell lines showed that 5-fluorouracil can alter profoundly miRNA expression patterns and miRNAs modulate the expression of key proteins involved in the regulation of cell proliferation, apoptosis and drug response[255,256]. In one of the first studies on CRC patients, miRNA expression was evaluated in cancer biopsies before therapy and two weeks after starting preoperative capecitabine chemoradiotherapy: miR-137 and miR-125b were upregulated two weeks after starting therapy and higher levels of both of them were associated with worse response to the therapy[257]. Hansen *et al*[258], studied the predictive value of miR-126 in clinical response to capecitabine and oxaliplatin in CRC metastatic patients by *in situ* hybridization. MiR-126 expression levels were significantly higher in patients responding to therapy with respect to not responding patients: furthermore, its high expression was associated with a more prolonged free survival progression[258]. Overexpression of miR-145 was found in CRC patients who showed a good response to neoadjuvant chemoradiotherapy (5-FU and 50.4 Gy) and tumor regression[259], while miR-17-5p was found increased in chemoresistant patients[260]. Della Vittoria Scarpati *et al*[261] using microarray expression profiling detected specific miRNA signatures associated with complete response of CRC patients after neoadjuvant chemoradiotherapy. Specifically, they found a set of 13 miRNAs (*i.e.*, 11 upregulated and 2 downregulated miRNAs) strongly associated with good response to chemoradiotherapy. Among them, the upregulation of miR-622 and miR-630 showed 100% sensitivity and specificity in predicting the patients' response[261]. In a similar study, Svoboda *et al*[262] compared miRNA profiles of CRC patients classified as sensitive or resistant to neoadjuvant chemoradiotherapy. They identified an overexpression of miR-215, miR-190b,

and miR-29b-2* in non-responder patients, while the upregulation of let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a* was associated with good responders[262]. In another profiling study, Hotchi *et al*[263] analyzed miRNA expression in rectal cancer patients prior to pre-operative chemoradiotherapy and correlated them to different methods to evaluate the patients' response. They found different miRNA signatures that discriminated responders from non responders. MiR-223 was the only miRNA common to the different evaluation parameters: its expression was significantly higher in responders compared with non-responders and showed 100% and 78% sensitivity and specificity, respectively, in the prediction of response to pre-operative chemoradiotherapy[263]. In the last few years some evidence on the potential involvement of lncRNAs in molecular mechanisms controlling cancer drug resistance has been reported. Cancer Upregulated Drug Resistant (CUDR) is a lncRNA upregulated in several cancers, including CRC. Enforced overexpression of CUDR induced resistance to doxorubicin and etoposide, as well as drug-induced apoptosis in human squamous carcinoma A431 cells[264]. Lee *et al*[265], by analyzing the expression of 90 lncRNAs in 5-FU-resistant CRC cell lines, found that SnaR and BACE1AS were significantly downregulated in resistant cell lines; decreased expression of SnaR was responsible for increased cell viability after 5-FU treatment. Recently, colorectal cancer-associated lncRNA (CCAL) was found significantly upregulated in CRC patients with worse response to adjuvant chemotherapy[266]. CCAL regulates CRC progression and multidrug resistance (MDR) through activation of the Wnt pathway by suppressing AP-2α and leading to upregulation of MDR1/P-gp expression[266].

CRC associated single nucleotide polymorphisms in non-coding RNAs

According to the multigenic model of cancer development, combinations of polymorphic genetic variants in susceptibility genes could contribute to CRC risk. Recently, functional polymorphisms in miRNAs or their binding sites in mRNA targets have been discovered in association with pathological phenotypes. A SNP embedded in a miRNA sequence may alter miRNA maturation or its targeting and, accordingly, contribute to the onset and evolution of cancer. One of the first studies showing a prognostic role of miRNA SNPs in CRC reported a significant association of SNP rs7372209 in pri-miR26a-1 to positive chemotherapy response, and SNP rs1834306 in the pri-miR-100 to longer time to progression, suggesting that miRNA polymorphisms could be potential predictors of clinical CRC outcome[267]. Xing *et al*[268], by screening seven SNPs in 408 CRC patients of a Chinese population, identified two SNPs statistically associated with prognostic features: rs6505162 in pre-miR-423 was correlated to the overall survival and recurrence-free survival, while, rs4919510 in pre-miR-608 was correlated with recurrence-free survival. CRC patients with both SNPs had a 2.84-fold increased risk of recurrence and/or death.These associations were evident only in patients receiving chemotherapy[268]. In another study, performed on 1,097 CRC patients recruited at the University of Texas MD Anderson Cancer Center, rs4919510 in miR-608 associated with increased risk for recurrence and death, and rs213210 in miR-219-1 in association with death of patients with stage Ⅲ disease were found[269]. Patients carrying both SNPs showed a 5.6-fold increased risk of death. SNP rs11614913 in miR-196a-2 was associated to a significantly increased CRC risk in a Korean population[270]. Association between rs11614913 and CRC susceptibility was also valuated in two different Chinese studies, but the results were conflicting[271,272]. Recently, a study on SNP rs895819 in pre-miR-27a, previously associated with different cancers, was performed to valuate the potential association to CRC susceptibility in a Han Chinese population: GG genotype was significantly associated with risk of CRC and metastasis[273].Data on association to CRC risk of previously mentioned SNPs in miR-196a-2 and miR-27a was not confirmed in a Central-European Caucasian population[274]. The discrepancies in the diagnostic potential of miRNA SNPs in CRC above reported may be due to different molecular pathogenetic mechanisms that differently contribute to cancer or population-specific factors, such as the different genetic backgrounds of the studied population. In addiction to the above-mentioned SNP rs6983267 mapping to a genomic region abundant in lncRNAs, five SNPs in the lncRNA PRNCR1 (prostate cancer associated non-coding RNA 1) were investigated in a Chinese case-control study of 313 cases with CRC and 595 ethnicity-matched controls[275]. The final results of this study were that rs13252298 and rs1456315 are associated with significantly decreased risks of CRC. Tumours of patients expressing rs1456315G were larger than 5 cm. Patients expressing rs7007694C and rs16901946G had a decreased risk of developing poorly differentiated tumours; on the contrary, expression of rs1456315G was found to be associated with an increased risk[275].

NON-CODING RNAS IN BODY FLUIDS: BIOLOGICAL AND DIAGNOSTIC IMPLICATIONS

Among the most ambitious goals of oncology is the application of fast and non-invasive methods for the identification of specific biomarkers, to be applied to diagnosis and prognosis (*i.e*., liquid biopsies): these could be useful to discriminate between healthy individuals and cancer patients or as markers of specific responses to therapy. In this regard, blood is the

election sample: it contains a high amount of important biomolecules, such as proteins, hormones, metabolites, DNA and different RNAs such as mRNAs and miRNAs. The stoichiometric ratio of its components is highly dynamic and reflects the health status of patients. Blood sampling is fast and easy: serum and plasma have been used for a long time as a source of biomarkers for different conditions. MiRNA profiling is an established method for discriminating cancerous tissues from their healthy counterparts[276]. The discovery, in 2008, of miRNAs in human plasma opened new intriguing perspectives in cancer diagnosis[277,278]. An important requisite of biomarkers is stability in the medium where they are released: miRNAs have been shown to be present in serum in a stable form that prevents their digestion by RNases. Little is known about the origin and features of serum miRNAs, but it is known that they are cell-free molecules: therefore, there have to be other ways to protect them from degradation[277]. Currently, two major release mechanisms of circulating miRNAs have been proposed: (1) active secretion of miRNA-containing shedding microvesicles or exosomes[279]; and (2) active secretion of miRNAs in the form of ribonucleoprotein complexes[280]. Exosomes have been described as a new mechanism of cell-to-cell communication that has similar features to hormonal signalling[281]. Exosomes originate from inward budding of endosomal membranes forming multivesicular bodies, which are secreted into the extracellular environment when late endosomes fuse with the plasma membrane[282]. These nanovesicles are produced by several cell types, both in normal and pathological conditions, and were found in serum and other body fluids[283-286]. Interestingly, exosomes have been shown to carry a set of miRNAs that are specifically targeted to them[281,287-289]. The endosomal sorting complexes required for transport (ESCRT) has been hypothesized to be major regulators of types and amounts of biomolecules sorted into exosomes (*i.e.*, miRNAs, mRNAs, proteins)[290]. The molecular mechanisms involved were uncharacterized until recently. The identification of the role of sumoylated hnRNPA2B1 in transfering miRNAs to exosomes is the first evidence of a possible mechanism involved in the specific miRNA sorting into exosomes[291]. Despite the great interest and growing research in the exosome field, accumulating data suggest that these nanovesicles contain only 10% of circulating miRNAs, and that most of them are associated with RBPs[292]. In 2011, Arroyo *et al*[293] found that the majority of circulating miRNAs are destabilized by plasma digestion with proteinase K, demonstrating that a protein complex is responsible for their protection in the RNase-enriched plasma environment. Notably, for the miRNAs that are not carried by microvesicles or exosomes, a strong association with Argonaute-2 protein was detected. Another mechanism putatively involved in miRNA preservation in serum is the packaging of circulating miRNAs in high-density lipoproteins (HDL), which in turn can transfer them to recipient cells: this would identify a potential new role for HDL particles in cell communication[294]. Whatever the origin of circulating miRNAs, their expression profiling has proven to have prognostic relevance, as confirmed by several studies. For instance, in 2008 it was shown for the first time that miRNA profiling of circulating exosomes in ovarian cancer patients could potentially be used as a non-invasive diagnostic method of this malignancy. Eight miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214), known to have a diagnostic value in ovarian cancer tissues, were shown to have the same expression pattern in the exosomes of cancer patients: this was different than that of healthy controls[295]. Moreover, it has been noted that tumour cells possess increased exosome-shedding properties compared with normal cells[295]. In 2009, Rabinowits *et al*[296] made similar observations in lung cancer patients; in addition, they showed that the serum of lung adenocarcinoma patients is highly enriched in exosomes compared with healthy patients. In 2014, Eichelser *et al*[297] showed that cell-free miR-101 and miR-373 can be used as breast cancer-specific markers, and that exosomal miR-373, whose expression is increased in Erb-b2 receptor tyrosine kinase 2 (ERBB2/HER2) - negative tumours, has a potential role as a blood-based biomarker for more aggressive tumours. High levels of exosomes in the plasma of CRC patients are secreted by poorly differentiated tumours and are associated with decreased overall survival[298]. Serum levels of exosomal let-7a, miR-21, miR-23a, miR-150, miR-223, miR-1229, and miR-1246 were significantly higher in primary CRC patients at any disease stage than in healthy controls; these miRNAs were significantly downregulated after surgical resection of tumours[299]. Interestingly, exosomes secreted by colon tumour cells play a key role in the recruitment of inflammatory $CCR6^{+}CD4^{+}Th17^{+}$ into tumour sites and subsequently promote tumour growth[300]. Ragusa *et al*[289,301] have demonstrated significant alterations of exosomal miRNA cargo in CRC cells following treatment with Cetuximab. The most upregulated miRNAs in exosomes from Caco-2 and HCT-116 cells perform a dual biological function: (1) negative regulation of proliferation or apoptosis induction in cancer cells; and (2) immunosuppression in B- and T- cells. In the last few years, several papers reported the discovery of circulating miRNAs with potential diagnostic or prognostic power in CRC patients (Table 5). The first study on plasma miRNA profiling, performed on 90 CRC patients and 50 healthy controls, showed the significant upregulation of miR-17-3p and miR-92a (both encoded by the miR-17-92 cluster). The authors suggested that elevated plasma levels of miR-92a were not associated with inflammation or other gastrointestinal cancers, but most likely were CRC-related[302]. Upregulation of miR-92a (together with miR-29a) in

Table 5 Diagnostic and prognostic circulating non-coding RNAs in colorectal cancer

Clinical feature	Oncogene ncRNAs	Tumor suppressor ncRNAs	PMID
Diagnostic	miR-17-3p, miR-92a, miR-29a, miR-18a, miR-19a, miR-19b, miR-15b, miR-335, miR-221, exo-let-7a, exo-miR-1229, exo-miR-1246, exo-miR-150, exo-miR-21, exo-miR-223, exo-miR-23a, miR-378, miR-21, CRNDE, miR-155, miR-200c, miR-210	miR-601, miR-760, miR-143	19201770, 19876917, 23267864, 22970209, 20880178, 24705249, 25547322, 22868372, 22393467, 24310813
Metastasis detection	miR-29a, miR-21		22018950, 23704278
Survival	miR-141, miR-221, miR-21		21445232, 20880178, 23704278
Tumour size	miR-21		23704278
Tumour stage	miR-141		21445232

Cancer-related phenotypes that may be predicted by expression analysis of plasma/serum circulating oncogenic and tumor suppressive non-coding RNAs (ncRNAs). Exo-miR: MiRNAs purified from exosomes.

plasma was also reported by Huang *et al*[303]. They strengthened these data showing that levels of both miR-29a and miR-92a were significantly reduced in post-operative plasma samples when compared with pre-operative samples. In a different study, the same authors found that the levels of plasma miR-601 and miR-760 were significantly decreased in CRC compared with healthy controls[304]. It is interesting to note that by adding expression data of miR-29a and miR-92a for combined receiver operating characteristic (ROC) curve analysis with miR-760, the resulting AUC (Area Under Curve) increased to 0.943 with 83.3% sensitivity and 93.1% specificity in discriminating CRC from controls: this suggests the additive effect in diagnostic power of these 3 miRNAs[304]. Overexpression of miR-29a was also detected in the serum of CRC patients with liver metastases, but not in CRC patients without metastases: this suggests its potential as a novel non-invasive biomarker for early detection of CRC with liver metastasis[305]. By genome-wide miRNA expression profiling on 196 plasma samples from 123 patients with sporadic CRC and 73 healthy individuals, Giráldez *et al*[306] found significant upregulation of 6 miRNAs (*i.e.*, miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335), suggesting specific expression patterns to be considered as biomarkers for CRC. Upregulation of miR-141 levels was demonstrated in plasma of CRC patients with stage Ⅳ with respect to patients with stages Ⅰ-Ⅱ and unaffected controls; they were also associated with shorter survival. These expression differences of miR-141 may be related to a differential inflammatory response in CRC patients[307]. Similarly, the expression levels of miR-221 in plasma of CRC patients are significantly higher than in the healthy controls. According to the Kaplan-Meier analysis, upregulation of miR-221 is a significant prognostic factor for poor overall survival in CRC patients: patients with higher miR-221 plasma levels had dramatically lower survival rates than those belonging to the low expression group[308]. Upregulation of miR-155, miR-200c, and miR-210 from serum of CRC patients was reported by Chen *et al*[309] Expression levels of these miRNAs returned to normal levels in patients with good prognosis after surgery and chemotherapy. Unsurprisingly, several authors reported high levels of miR-21 in plasma and serum of CRC. Upregulation of plasma miR-21 discriminates CRC patients from controls with 90% specificity and sensitivity[310]. MiR-21 levels are significantly elevated in preoperative serum from patients with adenomas and CRCs. High miR-21 expression in serum is statistically associated with tumour size, distant metastases, and poor survival[311]. Recently, Monzo *et al*[312] showed that miR-21 expression was higher in plasma from blood drawn from the mesenteric veins (MV), near the site of primary CRC than in that drawn from the peripheral veins (PV) of the same CRC patients. Moreover, high levels of miR-21 in MV plasma were associated with shorter disease-free survival of patients. These data suggested that CRC cells release high concentrations of miR-21 in the MV, but that these concentrations are progressively diluted in the peripheral circulatory system. Accordingly, miR-21 dosage from MV would be a stronger prognostic CRC marker than from PV. Recently, Clancy *et al*[313] analyzed miRNA expression in serum of CRC patients in a literature review on about twenty different published studies: they reported that the 6 circulating miRNAs most frequently found to be dysregulated in CRC are miR-18a-5p, miR-21-5p, miR-29a-5p, miR-92a-5p, miR-143-5p and miR-378-5p. Recently, there has been a notable increase of reports on serum or plasma lncRNAs, potentially useful as biomarkers for different types of tumours. One of the first studies associating circulating lncRNA expression to specific cancers reports the upregulation of lncRNA HULC in blood samples of hepatocellular carcinoma patients[252]. The lncRNA H19 has been found to be highly expressed in the plasma of gastric cancer patients compared with healthy controls, and its levels significantly decrease after tumour resection[314]. Recently, a three lncRNA [urothelial cancer associated 1 (UCA1/CUDR), long stress-induced non-coding transcript 5 (LSINCT-5), phosphatase and tensin homolog pseudogene 1 (PTENP1)] signature in serum was identified as a diagnostic marker for gastric cancers[315]. Tong *et al*[316] identified the lncRNA POU3F3 (POU class 3 homeobox

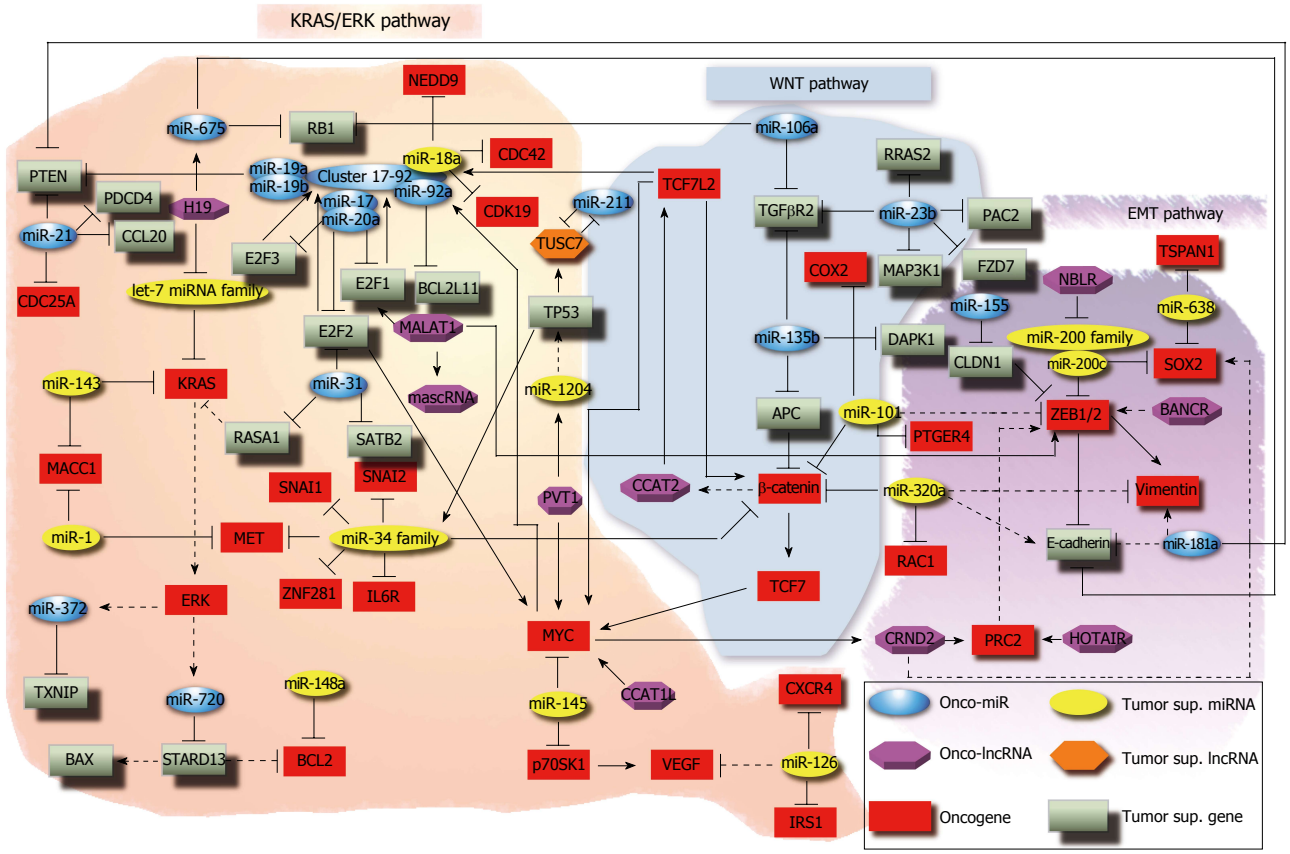

Figure 1 Non-coding network of colorectal cancer. Molecular signalling of non-coding RNAs [*i.e.*, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)] in most common cancer-related pathways involved in CRC etiopathogenesis (*i.e.*, KRAS/ERK, WNT, and EMT). RNA-RNA and RNA-protein interactions were retrieved from literature cited in this review. Lines with arrowheads represent expression activation, those with bars represent expression inhibition. Dotted lines show indirect interactions.

3) as a novel serum biomarker for esophageal squamous cell carcinoma, showing that combining POU3F3 expression with plasma levels of the squamous cell carcinoma antigen notably improves efficiency of screening for early detection. Quite surprisingly, only one report has been published to date on the expression of circulating lncRNAs as potential non-invasive diagnostic markers in CRC. The expression level of CRNDE-h transcript in plasma of CRC patients was significantly higher than that of healthy controls, with a sensitivity of 87% and specificity of 93% for diagnosing CRC[233] (Table 5).

FROM A COMPLEX NON-CODING RNA NETWORK TO NEW THERAPEUTIC TARGETS

We have discussed the multiple lines of evidence that pinpoint ncRNAs as key regulators of global gene expression for cancer-related processes. NcRNAs appear to constitute multiple control layers that define the correct functioning of classically known coding layers in cells: they perform this job by modulating chromatin architecture and genome expression (RNA synthesis, stability, processing and translation). The existence of this sophisticated RNA-based regulatory system has important biological and biomedical consequences. Indeed, it could appropriately explain the diversity of phenotypes of mammals, despite their relatively similar proteomes. On the other hand, it is becoming increasingly clear that alterations of these regulatory RNA signals may cause cells to acquire a pathological phenotype, including cancer, as reported for CRC in this review. Both miRNAs and lncRNAs contribute to bring about dysfunctions of critical CRC signalling pathways, such as KRAS/ERK, WNT, and EMT (Figure 1). RNA-based regulation in CRC constitutes a complex, and only partially explored, network of RNA-RNA, RNA-protein interactions that performs a multilayered control on proliferation, invasion, and differentiation in CRC cells. The complexity of this scenario is increased by the presence of several positive and negative regulatory loops. For instance, the miRNA cluster 17-92 represses E2F2, which in turn transcriptionally controls MYC. On the other hand, MYC acts as a transcriptional activator for the cluster 17-92[317]. Moreover, several ncRNAs act as mediators in the cross-talk between different molecular processes in CRC. For instance, MALAT1 induces the expression of E2F2, thus altering MYC signalling and increasing proliferation; it also activates the expression of ZEB1,

causing the impairment of epithelial differentiation. Indeed, these apparently different non-coding landscapes converge into a unique RNA network that pervades and controls protein-coding patterns. This key role of ncRNAs makes them very attractive and promising targets for innovative therapeutic approaches. Compared with other strategies, RNA-based therapeutics have several advantages. RNAs are molecules more "druggable" than proteins, because their targeting is mainly based on nucleic acid complementarity, and therefore easy to design and inexpensive to synthesize. RNA-based therapeutic approaches can be divided into two different categories: (1) RNA inhibition therapy, when target RNA is overexpressed; and (2) RNA replacement therapy, when the ncRNA is repressed (this is more feasible for miRNAs). A widely used method for miRNA inhibition is the use of chemically modified oligonucleotides complementary to mature miRNAs (antagomiRs). AntagomiRs quench target miRNAs, which may induce miRISC disruption and miRNA degradation[318]. MiRNA sponges with multiple complementary 3'UTR mRNA sites have been developed to inhibit the activity of miRNA families sharing a common target sequence[319]. Another approach to specifically inhibit miRNA function is to exploit miRNA masks (miR-Masks): these are complementary to the binding sites in the 3'UTR of the target mRNA, so that the miRNA is no longer able to perform its repressive activity[320]. MiRNA replacement therapy is based on the restoration of tumour suppressive miRNAs, expressed at lower levels in cancer. This can be obtained by inserting oligonucleotide mimics, containing the same sequence as the mature endogenous miRNAs, into the cells; alternatively, synthetic pre-miRNA mimics can be used[321,322]. The depletion of oncogenic lncRNAs could be obtained by using similar approaches to those applied to miRNAs: siRNAs and shRNAs (small hairpin RNAs) exhibit great RNA selectivity and knockdown efficiency and utilize a similar silencing molecular mechanism (RISC)[323]. However, in the last few years new appealing methods have been developed, which have the potential to substitute RNAi-based approaches [*i.e.*, antisense oligonucleotides (ASOs), ribozymes and aptamers]. ASOs are single stranded DNAs or RNAs (8-50 nt) with sequence complementary to their target RNAs[324]. When ASOs bind to target RNAs, RNase H1 recognizes the DNA:RNA/RNA:RNA heteroduplexes and catalyzes the cleavage of the RNA molecules[324]. Because ASO-mediated silencing is independent from the RISC machinery, this technology produces less off-targeting effects than siRNAs. As a consequence of their double-stranded structure, siRNAs and shRNAs may trigger an innate immune response and induce high levels of inflammatory cytokines and a toll-like receptor (TLR)-mediated response; such effects are less evident for single-stranded molecules[325]. Ribozymes are naturally synthesized RNA molecules that are able to degrade other RNAs by their intrinsic catalytic functions: their potential application as a therapeutic cancer tool has been already mentioned[326]. Ribozymes bind their targets by complementarity and catalyze cleavage of target RNAs, destabilizing their phosphodiester backbone[326]. An important hurdle to antisense-based lncRNA modulation is the presence of extensive secondary structures along these RNA molecules. Indeed, the effectiveness of siRNAs/shRNAs, ASOs and ribozymes could be seriously impaired by secondary structures in target RNAs[327]. This obstacle could be overcome by aptamers as their targeting mechanism is independent from sequence complementarity[328]. Aptamers are short DNA or RNA oligonucleotides or peptides with a stable three-dimensional structure that specifically bind to their targets (*i.e.*, RNAs, proteins, and small molecules) by fitting the three-dimensional shape of their ligands[329]. Aptamers would be able to recognize distinct RNA structures through tertiary interactions and alter their functions by sequestering them or masking potential binding sites. These RNA-based therapeutic strategies applied to ncRNAs have already shown very promising results in cancer, including CRC. The reintroduction of miR-145 has been demonstrated to perform an antitumoral role in a mouse model of colon cancer[321]. *In vivo* depletion of miR-21 by ASOs strongly inhibits pancreatic ductal adenocarcinoma tumour growth by inducing cell death[330]. Targeting MALAT1 and HOTAIR with ASOs in mouse xenograft models prevented lung metastasis and inhibited pancreatic tumour growth, respectively[331,332]. However, the main issue related to ncRNA therapeutics is to find a delivery mechanism that would allow their stability in the circulation and efficient tissue-specific uptake, at the same time minimizing off-target side effects[333]. Rapid progress in drug delivery systems has provided optimistic perspectives for advances in this field[334]. Different chemical groups, such as steroids and cholesterol, can be covalently added to miRNAs or ASOs to improve intracellular uptake and extend circulation time[335]. Further delivery mechanisms are the use of adenoviral vectors as carriers of RNA therapeutics, cationic liposomes, or polymer-based nanoparticles, which are able to form micelle-like structures[336]. Recently, an exosomal-based miRNA delivery has been reported to be an effective therapeutic delivery system because these nanoparticles are less toxic and better tolerated by the organism. Moreover, they are naturally used by cells for intercellular communication, and have been proven to protect their molecular cargo in the circulation[337]. For the development of RNA therapeutic strategies, it is interesting to consider that several biological functions of miRNAs and lncRNAs may be partially redundant (Figure 1): accordingly, it may be quite difficult to modulate them by targeting a single ncRNA molecule[338]. This should lead researchers to develop multitargeted RNA therapies in order to increase their impact on oncogenic molecular signalling. For instance, to attenuate KRAS overfunctioning in CRC, exogenous

let-7 miRNAs could be introduced to repress KRAS expression; concomitantly, ASOs against lncRNA H19 could be applied to deplete its action as a molecular sponge of the let-7 family. This would strengthen the antiproliferative effects triggered by KRAS repression. Such synergic approaches have already provided very encouraging results by simultaneously administrating miRNA mimics, siRNAs and shRNAs against mRNAs in *in vitro* and *in vivo* models[339]. Finally, despite the high number of putatively "*druggable*" ncRNAs, it is clear that researchers still have to overcome many conceptual and technical challenges to develop effective anticancer strategies that can be applied to patients. However, the results obtained so far are promising and provide optimistic perspectives for the future of anticancer treatments.

FUTURE PERSPECTIVES ON THE CLINICAL UTILITY OF NON-CODING RNAS

Experimental evidence of the potential predictive and prognostic value of cellular and circulating ncRNAs strongly suggests the possibility of exploiting them as useful clinical tools for cancer management. Expression changes of specific ncRNAs can be detected in CRC tissues, utilizing slide-based staining assays: these are standard diagnostic procedures in clinical laboratories. Analysis of plasma or serum ncRNAs could detect tumour biomarkers through PCR at the preoperative stage, indicating their value as early-diagnostic tools. Notwithstanding this, only few reliable diagnostic tests, based on dosage of ncRNAs, have been developed and commercialized to date (*e.g.*, the miRNA-based diagnostic tests of Rosetta Genomics). This slow clinical translatability could be due to different biological and technical factors. Many pre-analytical and analytical factors, including those that are donor/patient-related, can interfere with accurate ncRNA quantification. Due to non homogeneous technical approaches or to real biological heterogeneity of different patient cohorts, expression profiles obtained by various research groups may be different. The real predictive power of these profiles has to be carefully investigated on extended cohorts of patients to understand their actual clinical significance; for instance, data reported on the prognostic value of cellular miR-31 in CRC seem to be very promising, but no univocal results have been obtained to date. Many researchers have compared different protocols for plasma/serum preparation and analyzed the differences between the two biological fluids in terms of amount of circulating miRNAs, but contrasting results have been obtained[277,340,341]. This inconsistency could be explained by the different methods used for the separation of plasma and serum from whole blood (such as different centrifugation protocols): these could lead to different amounts of blood cell contamination[342,343]. However, the effective tumour-specificity of ncRNAs as blood biomarkers remains to be substantiated. Many circulating ncRNAs, whose increased expression had been associated to CRC, could also be related to the presence of other neoplastic diseases. In three independent meta-analysis studies, the diagnostic value of circulating miR-21 in CRC was verified: the authors reported that miR-21 has a moderate potential diagnostic value for CRC[344-346]. However, several papers on different types of carcinoma have also proposed circulating miR-21 as a diagnostic marker of cancer[347-349]. Accordingly, the common upregulation of miR-21 in the blood of patients with different types of cancers makes it a poorly specific diagnostic marker. In fact, Wang *et al*[350] analyzed the value of circulating miR-21 and reached the conclusion that it might be considered a relevant prognostic biomarker in general carcinomas, but not a sensitive specific diagnostic biomarker. The same observations could also be applied to other CRC-related circulating RNA molecules, commonly found in patients with different tumours or other pathological conditions (*e.g.*, miR-17, miR-18a, miR-20a, miR-29a, and miR-92a)[351-354]. Definitively, all these considerations would suggest that ncRNA-based diagnostic approaches have to be greatly improved. CRC screening should be based on a complex panel of different RNA species (both miRNAs and lncRNAs), rather than on a single or few RNAs. Simultaneous dosage of multiple RNAs having complementary discriminatory and prognostic power could represent a powerful predictive tool. This approach could be improved by analyzing the ratio of expression levels among different ncRNAs (as combined biomarkers), rather than evaluating the concentrations of single RNAs[348,355,356]. Finally, the use of ncRNA-based assays will open new and interesting fields in the screening and monitoring of cancers, including CRC. However, many issues must be addressed before these findings can be translated into a clinically useful screening strategy for CRC patients.

CONCLUSION

The literature on ncRNAs in CRC has grown considerably over the last decade, confirming their role in this neoplasia. Based on this huge amount of data, there is no doubt that ncRNAs will be important biomarkers in cancer diagnosis and prognosis, as well as therapeutic targets for the treatment of CRC[357]. However, before ncRNAs are routinely applied to clinical settings, it will be critically important to activate large collaborative efforts to fully realize the clinical potential of this approach. Moreover, a deeper knowledge on ncRNA molecular targets, together with new selective methods for RNA delivery to cancer cells, would lead to great benefits for the treatment and management of CRC. NcRNA biomarkers and RNA-based therapies are very promising tools and approaches to supplement current

diagnostic/prognostic and therapeutic strategies for CRC.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Neuroendocrine differentiation: The mysterious fellow of colorectal cancer

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Abstract

Neuroendocrine differentiation in sporadic colorectal cancer has been recognized since decades, but its clinical impact is still controversially discussed. Detailed parameter analyses hint at the possibility that probably not neuroendocrine differentiation itself, but its association with poor grade of tumor differentiation, lymph node metastases, distant metastases and other unfavorable features contribute to worse clinical outcome. However, other studies deny a relationship between neuroendocrine differentiation and prognosis of colorectal cancer. This review elucidates, whether new insights into the origin of neuroendocrine differentiation in the intestinal epithelium, its regulation by mTOR pathway components and its possible link to the intestinal stem cell compartment could determine a role of neuroendocrine cells as prognostic marker and putative therapeutic target in sporadic colorectal cancer.

Key words: Neuroendocrine differentiation; Colorectal cancer; mTOR pathway; Neuroendocrine tumorigenesis; Targeted therapy

Core tip: Neuroendocrine differentiation in sporadic colorectal cancer has been recognized since decades. In contrast to the clinico-pathologically well-defined pure neuroendocrine tumors and mixed adenoneuroendocrine carcinomas of the colon and rectum, the clinical impact of focal neuroendocrine differentiation in colorectal carcinomas is still controversially discussed. Further insights into the regulation of neuroendocrine differentiation by mTOR pathway components and recent knowledge about a link of enteroendocrine cells to the intestinal stem cell compartment hint at a role of neuroendocrine cells as prognostic marker and putative therapeutic target in sporadic colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide[1]. More than 50% of patients with CRC experience recurrence or metastases despite of curative operations[2]. Cytotoxic drugs applied as monotherapy or combined with monoclonal antibodies targeting proangiogenic vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) have been shown to prolong survival[3,4]. However, a proportion of patients gain little or even no benefit from these therapeutic regimens[5]. These facts underline the need to increase knowledge about special phenotypes, somatic genetic alterations and signaling pathways, which can be translated into prognostic markers or new molecularly defined targets for therapy of CRC.

The recent approval of new drugs for the treatment of advanced pancreatic neuroendocrine tumors brought neuroendocrine differentiation of tumor tissue, also beyond the pancreaticobiliary tract, into the focus of oncologists[6-8]. Whereas pure neuroendocrine tumors (NET) of the gastrointestinal tract have been established as well defined entities, the prognostic and therapeutic relevance of neuroendocrine differentiation in sporadic colorectal cancer has been less extensively evaluated. Insights into the histogenesis, epidemiology and pathogenetic links to known cancer pathways are necessary to elucidate the sufficiency of neuroendocrine cells as prognostic marker or new target for the therapy of CRC.

ORIGIN OF ENTEROENDOCRINE CELLS

Enteroendocrine cells comprise approximately 1% of epithelial cells in the gastrointestinal system and represent the largest population of hormone-producing cells in the body[9].

Detailed investigation revealed that enteroendocrine precursor cells differentiate immediately from self-renewing Lgr5+ intestinal stem cells[10]. This lineage differentiation depends on a regulatory cascade involving Notch signaling[10], the hairy enhancer of split (HES) transcription repressor and proendocrine basic helix-loop-helix (bHLH) transcription factors[11]. Expression of proendocrine bHLH factors, which is positively influenced by inactivation of Notch signaling[11], enables cells to differentiate toward divergent subsets of mature hormone-producing endocrine cells[12,13]. Several key transcription factors are involved in the regulation of enteroendocrine cell differentiation[14], which takes place within the crypts. Completely differentiated enteroendocrine cells are mainly determined to migrate upward along the villus as mature hormone-producing cells[12,13]. However, a small population of enteroendocrine cells migrates downwards to the bottom of the crypt or stays localized at the crypt base[15], where they reside in a Wnt signaling active zone and express both stem and postmitotic mature endocrine cell markers[16]. In both, intestinal and enteroendorine cell populations, expression of stem cell markers and continuous exposure to Wnt signaling could be hallmarks of cells, which are susceptible to neoplastic transformation[16,17].

However, data from a mouse model indicate that probably not these terminally differentiated enteroendocrine cells, but their early precursors respond to abnormal Wnt signaling by developing serotonin-expressing adenomas of the small intestine[18]. The concept that exocrine and endocrine components of CRC have the same cellular origin is supported by studies on mixed adenoneuroendocrine carcinomas (MANEC) and neuroendocrine carcinomas with minor associated exocrine components, which could demonstrate that both components share somatic mutations in several genes as *APC, TP53, DCC, KRAS, BRAF, ATM, CTNNB1, ERBB4, JAK3, KDR, RB1, BCL9, FOXP1*[19-22] and display identical LOH pattern on different chromosome loci as 5q, 17p, 18q[23]. Evidence of an additional mutation (*SMARCA4*)[22] or LOH involving 6q, 11p, 18q, *APC* marker and chromosome 3[23] in the endocrine tumor cell population could indicate that this component corresponds to a higher grade transformation of the tumor[20].

According to these data from mouse and human models, higher grade tumor transformation *via* development of neuroendocrine compartments can occur in every stage of intestinal or colorectal carcinogenesis and, furthermore, it could be even therapy-related. Probably both, pre- and postoperative as well as cytotoxic and radiation therapy could induce trans-differentiation in carcinomas with completely developed phenotype as indicated by the finding of increased neuroendocrine cells in a subset of distant metastatic compared to primary colorectal carcinomas[24] and in neoadjuvant treated compared to untreated rectal carcinomas[25]. This trans-differentiation from non-neuroendocrine to neuroendocrine tumor cells has already been proven for prostate cancer in an androgen-deprived environment[26,27] and is essentially associated with the phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) pathway[28]. Studies on both experimental and human sporadic neuroendocrine tumors (NETs) and on familial syndromes, in which NETs arise, point to the involvement of mTOR pathway components in neuroendocrine tumorigenesis in general[29]. Proven or putative activators of the mTOR pathway are mutations in upstream regulators of mTOR (*PTEN* and *TSC2*) and overexpression of a microRNA (miR-21) that targets PTEN and reduces

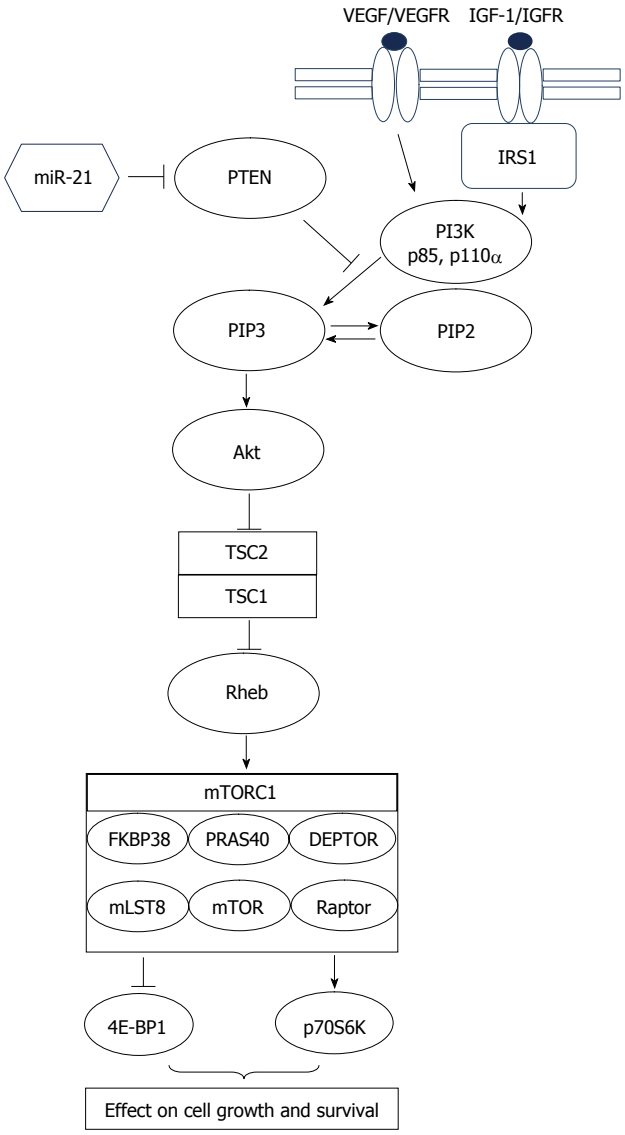

Figure 1 The PI3K/PTEN/Akt/mTOR-cascade (Modified according to Cingarlini *et al*[29] and McCubrey *et al*[32]). Phosphatidylinositol-3-kinase (PI3K) is a heterodimeric protein with an p85-kDA regulatory subunit and a p110α-kDa catalytic subunit (PIK3CA). PI3K phosphorylates membrane phospholipids, thereby forming the second messenger lipids phosphatidylinositol 3,4-biphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate (PIP3). Pleckstrin-homology (PH) domain of kinase Akt binds to PIP3, thereby promoting activation of Akt *via* phosphorylation by phosphotidylinositide-dependent kinase 1 (PDK1, not displayed in the Figure). Akt inhibits tuberous sclerosis 2 (TSC2 or tuberin) function through direct phosphorylation. TSC2 phosphorylation by Akt represses activity of the TSC1/TSC2 complex, allowing the small GTPase Rheb to activate the protein kinase function of mTOR (mammalian target of rapamycin). mTOR forms the catalytic core of the mTORC1 complex, which comprises additionally Raptor (Regulatory associated protein of mTOR) adaptor protein, DEPTOR (DEP domain containing mTOR-interacting protein), mLST8 (member of the Lethal-with-Sec-Thirteen gene family), FKBP38 (FK506 binding protein 38), and PRAS40 (proline-rich Akt substrate 40 kDa protein). Activated mTOR phosphorylates p70S6K, which induces a negative feedback loop uncoupling IRS-1 (insulin receptor substrate-1) from PI3K, thus preventing further signal transduction through this pathway. Negative regulation of the PI3K pathway is primarily accomplished though the PTEN tumor suppressor protein, which dephosphorylates phosphoinositide substrates as PIP3. Expression of PTEN is regulated by microRNA miR-21.

its expression[29-31] as displayed in Figure 1 (modified according to Cingarlini *et al*[29] and McCubrey *et al*[32]). The association between neuroendocrine trans-differentiation and mTOR pathway could be important for new targeted therapy regimens as discussed later in this review.

NEUROENDOCRINE DIFFERENTIATION IN COLORECTAL TUMORS: DIAGNOSIS, CLASSIFICATION AND EPIDEMIOLOGY

Neuroendocrine cells in non-neoplastic and neoplastic tissue of the gastrointestinal tract and nerve elements express a panel of identical antigens, which are used as neuroendocrine markers[33]. The markers synaptophysin, chromogranin A, B and C, HISL-19, neuron-specific enolase (NSE), the proprotein convertases PC2 and PC3, the lymphoreticular epitope Leu-7, and the neural cell adhesion molecule (or CD56) are sufficient to reveal neuroendocrine differentiation[33,34] independent of hormone production[35]. An example for synaptophysin expression in a colorectal adenocarcinoma is displayed in Figure 2. In addition, syntaxin1, VAMP2, SNAP25, alpha/beta-SNAP[36] and L-dopa decarboxylase (DDC)[37] have been used as neuroendocrine markers. In the pre-immunohistochemistry era, the Churukian-Schenk argyrophil stain[38] and the Grimelius stain[39] were applied to demonstrate neuroendocrine cells, which are argyrophilic. The currently known 15 neuroendocrine cell types of the gastrointestinal tract and pancreas produce different hormones, but all of them express the general neuroendocrine marker synaptophysin[40].

In accordance with the consensus guidelines of the European Neuroendocrine Tumor Society (ENETS)[41,42], the current WHO classification for gastrointestinal neuroendocrine tumors[43] applies a grading system based on mitotic activity and the percentage of Ki-67 labeled proliferating cells: Grade 1, grade 2 and grade 3 (= neuroendocrine carcinoma) are defined by mitotic counts of < 2/10 high power fields (HPF), 2-20/10 HPF and > 20/10 HPF, respectively, and/or by Ki-67 indices of $\leqslant$ 2%, 3%-20% and > 20%, respectively. A fourth group, mixed adenoneuroendocrine carcinoma (MANEC) is morphologically recognizable as both gland-forming epithelial and neuroendocrine phenotype, with each component representing at least 30% of the lesion[43]. An additional category considering neuroendocrine differentiation less than 30%, but above the level reported for normal colorectal epithelium (> 1 cell/mm^2[44,45], > 2%[36]) similar to that proposed previously by Jansson *et al*[46] has not been defined by the current WHO classification[43]. However, a detailed study on colorectal tumors with mixed glandular-neuroendocrine differentiation[47] revealed that also neuroendocrine tumor components comprising less than the currently

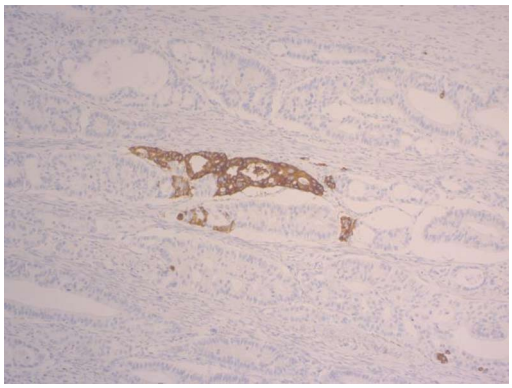

Figure 2 Primary colorectal cancer with focal synaptophysin expression (magnification × 200).

used 30% cut off could have negative impact on the clinical course and patient outcome, which sets the occasional finding of isolated neuroendocrine cells in colorectal cancer into a new focus.

In sporadic colorectal cancer, neuroendocrine cells have been identified in 8%-77.5% of cases[24,25,36,38,39,45,48-63], largely depending on the method used to assess the neuroendocrine cell population[24]. Neuroendocrine differentiation occurs also in hereditary non polyposis colorectal cancer (HNPCC, 51.4%)[64].

HISTOPATHOLOGICAL AND CLINICAL FEATURES OF SPORADIC COLORECTAL CANCER WITH NEUROENDOCRINE DIFFERENTIATION

Chromogranin A and synaptophysin are the most frequently used markers to study the link between neuroendocrine differentiation and clinicopathological characteristics. These markers are expressed in divergent patterns: Co-expression of chromogranin A and synaptophysin[63,65] occurs as well as predominance of one marker concomitant with absence of the other marker[65,66].

Studies focusing on the relationship between occasional neuroendocrine differentiation (*i.e.*, < 30%) and clinicopathological parameters in sporadic colorectal cancer are summarized in Table 1[24,25,39-71]. Detailed parameter analyses hint at the possibility that not neuroendocrine differentiation itself, but its association with poor grade of tumor differentiation, lymph node metastases, distant metastases and other unfavorable features contribute to the worse prognosis of sporadic CRC with neuroendocrine differentiation, which has been reported by several authors (Table 1). However, other authors deny a relationship between neuroendocrine differentiation and the prognosis of CRC (Table 1).

The assumption of a causal relationship between neuroendocrine differentiation and tumor differentiation is growing stronger after introduction of a new histologic grading system based on the number of poorly differentiated cell clusters (PDC) in CRC[72,73]. In a study of 20 consecutive CRCs with high grade PDCs (≥ 10 clusters, grade Ⅲ CRCs), the PDCs, but not the glandular part expressed synaptophysin[74]. This could be the morphologic correlate for the previously discussed "trans-differentiation", which initiates the development of a more aggressive tumor[20].

The presence of neuroendocrine cells in the proliferative compartments of gastrointestinal adenocarcinomas is well-documented[38,63]. Moreover, according to recent knowledge obtained from an adult Drosophila midgut model, enteroendocrine cells could function as local regulators of intestinal stem cell proliferation through modulation of the mesenchymal stem cell niche[75]. These findings could hint at the importance of neuroendocrine cells for both, maintenance and progression of tumors, thus contributing to the development of CRC with high survival potential and aggressiveness.

Further evidence for an indirect impact of neuroendocrine cells on the clinical outcome of CRC is given by the previously published link between chromogranin A/antioxidant enzyme co-expressing CRC cells and unfavorable prognosis, probably due to activated antioxidant defense and higher metabolic activity of the tumors[76]. In addition, high expression of MTOR or its downstream targets p-RPS6KB1, p-RPS6 and p-EIF4EBP1 is associated with adverse clinical outcomes in neuroendocrine tumors[77], but this link has not been investigated for neuroendocrine foci within sporadic CRC.

NEUROENDOCRINE DIFFERENTIATION AND THERAPY OF SPORADIC COLORECTAL CANCER

Neuroendocrine differentiation has been recognized as result of therapy and is getting into the focus of oncologist as target for new treatment approaches.

Shia *et al*[25] demonstrated an increased frequency and density of cells with an endocrine phenotype in rectal adenocarcinomas treated with neoadjuvant radiochemotherapy and found that the extent of endocrine cells appeared proportional to the degree of treatment response. This therapy-related endocrine differentiation of tumor cells could be induced by cytotoxic insult[25].

The approval of new drugs for the treatment of advanced pancreatic neuroendocrine tumors[6-8] harbored the possibility to extend the therapeutic spectrum also for neuroendocrine differentiated tumors beyond the pancreaticobiliary tract. Everolimus plus octreotide long-acting repeatable (LAR) showed significant benefits and improved outcomes for patients with advanced colorectal neuroendocrine tumors[78].

Table 1 Relationship between neuroendocrine differentiation and clinico-pathological parameters

Clinical parameter	Link to neuroendocrine differentiation	Ref.
Age	No	[39, 55, 63]
Gender	No	[39, 55, 63]
Preoperative conditions	Association with lower CEA levels	[63]
Tumor markers		
DNA ploidy	No	[50]
TP53 expression	No	[50, 67]
	Similar abnormal expression as in conventional adenocarcinomas	[25]
BCL-2 expression	Yes	[68]
Ki-67 labeling index	Very low (< 5%)	[25]
	No	[24]
Tumor localization	No	[24, 25, 39, 45, 48-50, 53, 55, 63, 66]
	Yes	[38, 59]
Tumor morphology		
Polypoid *vs* ulceration	No	[55, 63]
Tumor differentiation	No	[24, 39, 45, 48-50, 52, 53, 63, 66]
	Yes	[38, 56, 57, 60, 69]
Tumor size	No	[55, 63]
Tumor stage	No	[24, 39, 45, 48-50, 53, 55, 66]
	Yes	[56]
Lymphatic and venous invasion	No	[55, 63]
Perineural invasion	No	[63]
Lymph node metastases	No	[55]
	Yes	[57, 58]
Distant metastases	Yes	[47, 51]
Clinical stage	No	[53, 55]
	Yes	[36]
Therapy response	Associated with better response to radiochemotherapy	[25]
Prognosis	No	[38, 44, 50, 53, 55, 62, 63]
	Better prognosis	[60]
	Shorter survival from time of metastasis	[24]
	More aggressive behavior	[48]
	Poor prognosis	[36, 39, 45, 46, 54, 56, 57, 59, 61 (stage Ⅱ), 66 (stage Ⅲ and Ⅳ), 70, 71]

Phase Ⅰ trials including CRC patients demonstrated a positive effect on stable disease for one of these drugs, everolimus, when it was combined with cetuximab[79] or with 5-fluorouracil/leucovorin (5-FU/LV) or with mFOLFOX6 (5-FU/LV + oxaliplatin)[80]. Everolimus inhibits the PI3K/PTEN/Akt pathway by connecting to the FK-506 binding protein 12 to block mTOR (mammalian target of rapamycin) activation[79]. Considering a possible importance of the PI3K-Akt-mTOR pathway for neuroendocrine differentiation[28], neuroendocrine cells within sporadic CRC could be the putative target for mTOR-inhibitor therapy (for example everolimus). The published pharmacodynamics trials[79,80] did not consider special CRC phenotypes. However, according to new insights into genotype-phenotype correlations, pretreatment histomorphological characterization of CRC could possibly help to increase efficacy of mTOR-inhibitor therapy.

CONCLUSION

A possible role of neuroendocrine differentiation as prognostic marker and therapeutic target in sporadic colorectal cancer should be further elucidated by large cohort studies.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Eicosanoid pathway in colorectal cancer: Recent updates

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Abstract

Enzymatic metabolism of the 20C polyunsaturated fatty acid (PUFA) arachidonic acid (AA) occurs *via* the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, and leads to the production of various bioactive lipids termed eicosanoids. These eicosanoids have a variety of functions, including stimulation of homeostatic responses in the cardiovascular system, induction and resolution of inflammation, and modulation of immune responses against diseases associated with chronic inflammation, such as cancer. Because chronic inflammation is essential for the development of colorectal cancer (CRC), it is not surprising that many eicosanoids are implicated in CRC. Oftentimes, these autacoids work in an antagonistic and highly temporal manner in inflammation; therefore, inhibition of the pro-inflammatory COX-2 or 5-LOX enzymes may subsequently inhibit the formation of their essential products, or shunt substrates from one pathway to another, leading to undesirable side-effects. A better understanding of these different enzymes and their products is essential not only for understanding the importance of eicosanoids, but also for designing more effective drugs that solely target the inflammatory molecules found in both chronic inflammation and cancer. In this review, we have evaluated the cancer promoting and anti-cancer roles of different eicosanoids in CRC, and highlighted the most recent literature which describes how those molecules affect not only tumor tissue, but also the tumor microenvironment. Additionally, we have attempted to delineate the roles that eicosanoids with opposing functions play in neoplastic transformation in CRC through their effects on proliferation, apoptosis, motility, metastasis, and angiogenesis.

Key words: Eicosanoids; Cyclooxygenase; Lipoxygenase; Inflammation; Colorectal cancer

Core tip: Eicosanoids are bioactive lipids generated from polyunsaturated fatty acids (usually arachidonic acid) through highly regulated enzymatic pathways in many different cell types. These molecules are effective in small amounts, and may act in an autocrine or paracrine manner to regulate some of the most important steps in the development of acute

inflammation and its resolution. Aberrant expression of the enzymes that help synthesize these bioactive lipids is frequently seen in diseases associated with chronic inflammation, including cancer.

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INFLAMMATION AND COLORECTAL CANCER

Tumors show characteristics of inflamed tissue, including the presence of immune cells within the tumor tissue[1]. Although the presence of leukocytes within tumors was initially thought to result from anti-tumor immune responses, a role for inflammation in tumorigenesis is now generally accepted. Epidemiologic and clinical studies indicate that in response to chronic inflammatory conditions, epithelial cells (transformed and/or normal) and tissue-resident immune cells locally secrete cytokines, chemokines, growth factors, and pro-inflammatory mediators that recruit inflammatory cells from the circulation into the tumor site[2]. Furthermore, immune cells that invade the local tumor microenvironment are phenotypically different from normal immune cells, and can maintain the inflammatory milieu and promote invasion and migration of the transformed epithelial cells[3].

Colorectal carcinoma (CRC) is one of the best examples of an inflammation-associated cancer[4]. During colorectal carcinogenesis, epithelial cells in the colon accumulate mutations, which lead to either inactivation or activation of certain target genes that provide a selective growth advantage. In turn, this results in the transformation of normal epithelium to an adenomatous polyp, and finally to invasive CRC. The transformed epithelial cells then acquire the ability to secrete inflammatory mediators that act on pro-inflammatory leukocytes, endothelial cells, and fibroblasts to establish a tumor-promoting reactive microenvironment. For example, when compared with the general population, epidemiological studies have shown a higher incidence of CRC in patients with a previous history of inflammatory bowel disease (IBD)[5]. It has also become evident that inflammation is a significant factor in the progression of tumors. The regular use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers mortality from sporadic CRC and suppresses adenoma growth in patients with familial adenomatous polyposis (FAP) and who inherit a mutation in the tumor suppressor APC gene[6]. Similar to other solid malignancies, pathological examinations of CRC tissue reveal the presence of innate immune cells, including neutrophils, mast cells, natural killer cells, and dendritic cells that are recruited to the tumor and suppress tumor growth and angiogenesis[7]. This phenomenon is called immune-surveillance, and assists in the early detection and elimination of transformed cells and preneoplastic aberrant crypt foci (ACF), which may progress to adenomas and adenocarcinomas in CRC. On the contrary, colorectal and colitis-associated tumorigenesis are associated with the presence of an inflammatory microenvironment that favors the inhibition of immune-surveillance and promotes a tolerogenic environment with the release of growth factors, thereby supporting further tumor growth[8,9]. In addition to paracrine signaling by growth factors, cytokines, chemokines, and oxygen radicals[10], bioactive lipids derived from polyunsaturated fatty acids (PUFAs) are among the earliest signaling molecules released in response to an injury or inflammatory stimulus. The role played by these small mediators in inflammation and its resolution has garnered a great amount of recent interest[11,12].

POLYUNSATURATED FATTY ACIDS

Polyunsaturated fatty acids (PUFAs) that can be metabolized to bioactive lipids include arachidonic acid (AA), linoleic acid (LA), linolenic acid (LNA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). AA is a 20C polyunsaturated fatty acid (20:4n-6) that is usually esterified to the second carbon in membrane phospholipids, and gives rise to a wide variety of lipid products termed eicosanoids. AA is also known as an n-6 fatty acid, signifying the position of the carbon with the first double bond when considering the terminal methylene carbon group as the first carbon. AA is derived from linoleic acid (LA, 18:2n-6), an 18C essential fatty acid, through the subsequent actions of desaturases and elongases located primarily in the liver. The release of AA from phospholipids in the outer nuclear membrane is achieved through the activity of phospholipases such as the calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$). After being released, the free fatty acid can be metabolized *via* enzymatic pathways including the cyclooxygenase (COX) and lipoxygenase (LOX) pathways to generate 2-series prostaglandins (PGs) and thromboxanes (Txs) (COX pathway) or 4-series leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) (LOX pathway)[11,13] (Figure 1). The eicosanoids are highly potent, short-lived molecules that act locally, and have been strongly implicated in a variety of cancers, including CRC.

Long-chain PUFAs such as EPA and DHA, commonly known as n-3 fatty acids, are extensively found in fatty fish, but are not efficiently synthesized in humans[14]. As these fatty acids are primarily obtained through the diet, increased consumption of fish oil can alter the fatty acid profiles of the plasma and cell membranes in a time and dose-dependent manner[15], primarily at the

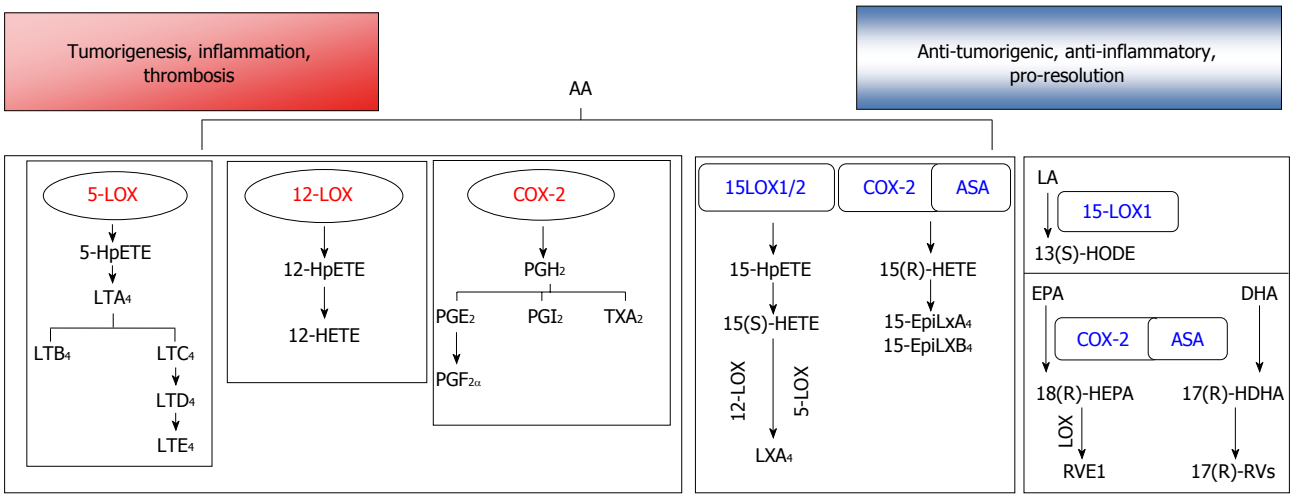

Figure 1 Enzymatic metabolism of polyunsaturated fatty acid can generate bioactive lipids that induce inflammation, tumorigenesis, and thrombosis, while also generating mediators with anti-tumorigenic, pro-resolution properties. In the pro-tumorigenic arm, arachidonic acid (AA) is metabolized *via* the cyclooxygenase (COX) pathway to generate prostaglandins (PGE_2, PGI_2) and thromboxanes (TxA_2). Lipoxygenase (LOX) enzymes convert AA to hydroxyeicosatetraenoic acids (HETEs), which are active on their own, or can be further converted to leukotrienes (LTs). In the anti-tumorigenic, pro-resolution arm, metabolism of AA through 15-LOX1/2 or acetyl salicylic acid (ASA) acetylated COX-2 generates intermediates that can be converted to lipoxins (Lxs) through the transcellular activity of other LOXs (5- or 12-LOX). Conversion of linoleic acid (LA) to 13(S)-HODE may produce anti-inflammatory effects mediated through activation of PPARγ. The fish oils eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may be converted by acetylated COX-2 to pro-resolution mediators E- and D-series resolvins (Rvs), respectively. PUFA: Polyunsaturated fatty acid.

expense of AA. This implies a decreased production of inflammatory AA-derived eicosanoids, which has been verified in healthy human volunteers who showed decreased levels of PGs and LTs after consuming EPA and DHA supplements for varying lengths of time[16]. EPA, being a 20C highly unsaturated fatty acid and therefore classified as an eicosanoid, can also be metabolized by the COX and LOX pathways into 3-series PGs and 5-series LTs. However, these lipids are readily recognized by PG and LT receptors, and are therefore considerably less potent in inducing inflammation[17]. Both EPA and DHA are substrates for the production of newly identified autacoids that are essential for the resolution of inflammation[18].

EICOSANOID PATHWAYS AND COLORECTAL CANCER

Bioactive lipids may modulate the incidence of cancer *via* several different mechanisms that include, but are not limited to, induction of inflammation, regulation of cellular oxidative stress, activation of receptors involved in cellular signaling pathways, and the alteration of membrane dynamics[19].

COX-2-derived lipid mediators

AA is metabolized to prostaglandins either by constitutively expressed COX-1 or by COX-2, which is expressed when induced by inflammatory stimuli[20]. *COX-2* is an immediate-early response gene that is not expressed in most cells, but is highly induced at sites of inflammation and in the tumor microenvironment[21]. The primary prostaglandin produced from AA (PGH_2) can be further metabolized to a several other prostaglandins; among which, PGE_2 has been strongly implicated in the development of gastrointestinal tumors[22]. This prostanoid acts *via* four G-protein coupled receptors (EP_{1-4}) and can enhance tumorigenesis through a variety of mechanisms, including enhanced cell proliferation, suppression of apoptosis, and induction of angiogenesis[23].

Elevated levels of COX-2 and an accompanying elevation of PGE_2 are often seen in CRC, and COX-2 expression is correlated with a lower survival rate among CRC patients[24]. It is well accepted that there are concerted interactions between carcinoma cells and other cells in the tumor microenvironment, and that these interactions contribute to cancer progression. PGE_2 modulates cancer-associated immune suppression by recruiting T cells, $CD8^+$ cytotoxic T cells, regulatory T cells, dendritic cells, and myeloid-derived suppressor cells (MDSCs)[20]. Additionally, secretion of PGE_2 may enhance oxidative stress, leading to a state of low grade continuous inflammation characterized by the infiltration of neutrophils and macrophages, and eventually leading to mitogenic signaling[18]. PGE_2 has been shown to stimulate macrophages to produce pro-inflammatory chemokines and cytokines, such as macrophage chemoattractant protein-1 (MCP-1), which recruits leukocytes from the circulation to local sites of inflammation[25]. A previous study[26] showed that MCP-1 levels were higher in intestinal epithelial cells, and that MCP-1 could stimulate COX-2 expression as well as the release of PGE_2 and vascular endothelial growth factor (VEGF) in human macrophages.

In the adaptive response, PGE_2 mediated signaling may affect cytokine production in antigen-presenting

cells by influencing the functional phenotype of T cells [*i.e.*, by switching the anti-tumor T helper 1 (Th1) responses to immunosuppressive Th2 responses)] during priming[27,28]. In trinitrobenzene sulfonic acid (TNBS)-induced colitis, a model of IBD, PGE_2 was shown to worsen inflammation and disease severity by increasing neutrophil and Th17 cell infiltration into colonic tissue[29]. Furthermore, PGE_2 was shown to amplify IL-23-mediated Th17 cell expansion by acting on its receptor (EP_4) located on T cells and dendritic cells[30].

The pro-tumorigenic effects of PGE_2 may also be mediated by T_{reg} cells, which contribute to immune evasion by tumor cells in a variety of cancers. Increased COX-2 expression and elevated PGE_2 production in adaptive FoxP3-positive T_{reg} cells found within tumors and mesenteric lymph nodes have been shown to contribute to an immunosuppressive microenvironment in CRC. This type of microenvironment facilitates tumor growth by suppressing effector T cells and inducing resistance to antigen-specific cancer immunotherapy[31,32]. A role for COX-2 in tumor immunity was also exhibited in COX-2 expressing colon cancer cell lines, where expression of FasL and TRAIL was shown to cause a counter-attack against cytotoxic T cells[33].

COX-2 overexpression is frequently associated with the concomitant expression of microsomal PGE synthase-1 (mPGES-1), the terminal synthase that leads to the preferential production of PGE_2[20,34]. Accordingly, in *Apc*-mutant mice, genetic deletion of mPGES-1 was reported to suppress intestinal cancer growth by reducing the size and number of aberrant crypt foci (ACF) in a carcinogen-induced colon cancer model[35]. Together, these findings suggest that mPGES-1 plays crucial roles during colon cancer progression, and that these roles are relevant to the promotion of inflammation. Thus targeting mPGES-1 may be a feasible option for cancer chemoprevention[36].

Interestingly, prostaglandins are also essential for the health of gastrointestinal mucosa by maintaining mucosal integrity, promoting wound healing, and limiting inflammation[36]. The absence of $cPLA_2$ in mice was recently shown to globally reduce the formation of AA-derived bioactive lipids, increase mucosal ulceration, and increase the expression of pro-inflammatory cytokines[34]. Mice with a targeted deletion of COX-2 in their endothelial cells and myeloid cells and treated with dextran sulfate sodium (DSS) displayed greater weight loss and had worse clinical scores compared to their WT littermates. However, mice with a targeted deletion of COX-2 in their colonic epithelial cells were not susceptible to DSS[37]. Additionally, mPGES-$1^{-/-}$ mice were recently reported to show more extensive inflammation in the GI tract when compared with WT mice[34]. This could have resulted from a shift in the metabolism of prostaglandins; *e.g.*, a loss of PGE_2 was associated with a gain in PGD_2, which has tumor suppressive functions[34,38].

Influx and efflux carriers such as the prostaglandin transporter (PGT) and multidrug resistance-associated protein 4 (MRP4), as well as the inactivation of prostaglandins (specifically PGE_2) by hydroxyprostaglandin dehydrogenase 15-(NAD) (15-PGDH) can regulate the availability and efficacy of prostanoids[20]. In fact, 15-PGDH is frequently down-regulated in a variety of cancers, suggesting its tumor-suppressive role[11]. Overexpression of 15-PGDH in colon cancer cells was shown to strongly inhibit tumor growth in immune-deficient mice. It was also demonstrated that colonic 15-PGDH expression was directly controlled and strongly induced by the activation of TGF-β, which has tumor-suppressive functions in colon cancer[39,40]. Therefore, the combined induction of COX-2 and inactivation of 15-PGDH in colon cancer can markedly increase PGE_2 levels, which may allow cancer cells to escape immune surveillance.

Randomized clinical trials and observational studies have indicated that long-term use ($\geqslant$ 5 years) of acetyl salicylic acid (ASA, Aspirin®), which inhibits both COX-1 and COX-2, leads to significant reductions in the risks for developing colorectal, esophageal, gastric, biliary, and breast cancer, as well as their distant metastases[41]. Furthermore, the daily use of ASA has been reported to specifically prevent the development of colorectal polyps and reduce the risk for developing sporadic CRC or CRC from Lynch syndrome[42-44]. Moreover, the use of ASA after a diagnosis of CRC can also improve survival, and especially in patients with COX-2-overexpressing tumors[24]. A recent prospective, observational study of ASA and COX-2 inhibitor use either during or after chemotherapy as an adjuvant in stage Ⅲ colon cancer patients reported reduced cancer recurrence and mortality[45]. Moreover, ASA use was associated with a greater reduction in the risk for developing colorectal tumors when the normal colonic mucosa showed a higher expression of 15-PGDH[45].

Other prostaglandins produced in the eicosanoid pathway, such as PGD_2, have been shown to down-regulate granulocyte infiltration into colonic mucosa at the early stages of TNBS induced inflammation[38,46]. More recently, it has been shown that mast cell-derived PGD_2 can function as an inhibitor of colitis and colitis-associated cancer (CAC) in mouse models[47]. Taken together, the findings suggest that different COX-2-derived prostaglandins can have opposing effects on inflammation, and that selective modulation of these mediators may prevent tumor growth in CRC.

LOX-derived lipid mediators

PUFAs are oxygenated *via* the enzymatic action of LOXs to form LTs and hydroxyeicosatetraenoic acids (HETEs), which both exert significant effects on the development and progression of human cancers[48]. Several different isoforms of LOX exist, including 5-LOX, 15-LOX-1, 15-LOX-2, and 12-LOX[13], and are named according to the position of the carbon atom in

AA that these enzymes oxygenate. Except for *ALOX5*, which is located on chromosome 10, most of the other LOX genes are located within a few megabases of each other on the short arm of chromosome 17[13]. Although AA is the preferred substrate for oxygenation for most LOXs, some LOX isoforms are capable of oxygenating fatty acids esterified to phospholipids or cholesterol[49,50].

In humans, 5-LOX is highly expressed in cells of myeloid origin, and especially in leukocytes[51]. This enzyme catalyzes the conversion of AA to 5S-hydroperoxyeicosatetraenoic acid (5-HpETE), and its subsequent conversion to LTA_4. LTA_4 can be converted to LTB_4 and then to cysteinyl leukotrienes by the actions of LTA_4 hydrolase and LTC_4, synthase, respectively[52] (Figure 1). 5-LOX activity is exquisitely sensitive to various stimuli, including the second messenger Ca^{2+}. Ca^{2+} can bind to the N-terminus of 5-LOX, which contains a hydrophobic domain and facilitates the binding of 5-LOX to membrane phospholipids[53]. The 5-LOX enzyme is usually located in the cytoplasm as a soluble protein; however, in the presence of Ca^{2+}, it may become phosphorylated and translocate to the nuclear or endoplasmic reticulum (ER) membrane, where with the help of 5-LOX activating protein (FLAP), it catalyzes the oxygenation of AA[53]. Therefore, many stimuli that increase the levels of intracellular calcium ions (*e.g.,* antigens, microbes, cytokines, and toxins) can induce the production of LTs[53,54].

LTs are classified into two general categories: LTB_4 and cysteinyl LTs (LTC_4, LTD_4, and LTE_4)[55]. LTs play key roles in the pathogenesis of inflammatory disorders, including IBD, and typically stimulate quick and short-lasting events (*e.g.,* smooth muscle contraction, phagocyte infiltration, increased vascular permeability), which are important in the pro-inflammatory context. These responses are mediated by G-protein coupled receptors: BLT1/2 for LTB_4, and CysLT1/2 and GPR17 for the Cys-LTs[52,56].

5-LOX is overexpressed in tissues with chronic inflammation and also in transformed cells. 5-LOX was shown to be up-regulated in patients with polyps and colon cancer[57], whereas in the $APC^{\Delta 468}$ mouse model of polyposis, the loss of 5-LOX was protective[58]. In the same model, 5-LOX metabolic products produced by hematopoietic cells were shown to promote tumorigenesis by enhancing both the proliferation of intestinal epithelial cells and recruitment of MDSCs to the spleen, mesenteric lymph nodes, and primary tumor[59]. Dietary administration of a 5-LOX inhibitor (Zileuton) to $APC^{\Delta 468}$ mice resulted in overall reductions in systemic inflammation, polyp number, and inflammatory infiltration into lesions[59].

Overproduction of LTB_4 in human colon cancer tissue is implicated in the pathogenesis of IBD. Additionally, high expression of the LTB_4 receptor BLT1 has been detected in human colon tissues[60]. These findings indicate the importance of an inflammatory autocrine loop during the promotion and progression phases of colon tumors. The inflammatory mediators can cause intestinal epithelial cells to up-regulate their expression of enzymes needed for the biosynthesis of eicosanoids, including the CysLTs, and signal transducing CysLT receptors, to provide a self-sufficient signaling mechanism needed to maintain both inflammation and tumor progression[58]. Taken together, these studies show that pro-inflammatory LTs might facilitate tumor growth by establishing an inflammatory microenvironment.

Metabolism of AA by 12-lipoxygenase (12-LOX) leads to the production of 12-HETE, which has been shown to stimulate the growth of various cancers[61]. Additionally, a $Gln^{261}Arg$ polymorphism in the *ALOX12* gene was shown to be associated with an enhanced susceptibility to several malignancies, which also indicates a potential oncogenic role for 12-LOX[62,63]. Although a recent meta-analysis of studies with 8379 subjects revealed that this specific polymorphism was not associated with an increased risk of colon cancer[64], other studies have reported that 12-LOX expression was associated with an oncogenic phenotype in CRC[62]. 12-LOX was also shown to be up-regulated in colon cancer specimens associated with inflammation[61]. Moreover, 12-LOX expressing colon cancer cell lines have demonstrated increased migration as a result of decreased E-cadherin and integrin-β1 expression[61], or enhanced production of reactive oxygen species (ROS) and activation of the catalytic subunit of the NADPH oxidase complex, Nox1[65].

Unlike 5-LOX and 12-LOX, 15-lipoxygenase-1 (15-LOX-1), which can oxygenate AA and LA as well as complex substrates such as biomembranes[66], may have an anti-inflammatory, tumor suppressive role in CRC. This enzyme can oxygenate AA to 15-HETE, or LA to 13(S)-hydroxyoctadecadienoic acid [13(S)-HODE]. Profiling of LOX metabolic products in CRC has shown that 13(S)-HODE was the only metabolite that significantly increased in the Caco-2 model of cellular differentiation[67,68]. Additionally, an assay of > 120 cancer cell lines from 20 different cancer types indicated an almost universal loss of 15-LOX-1 expression in de-differentiated cell lines when compared with well-differentiated cancer cells or non-transformed cells[68]. Moreover, levels of 13(S)-HODE were shown to be reduced in colorectal polyp samples obtained from patients suffering from FAP when compared to their levels in paired normal tissues[67]. A loss of 15-LOX-1 expression is primarily due to epigenetic factors, such as nucleosomal remodeling and the histone deacetylase (NuRD) complex[69]. Re-expression of 15-LOX-1 has been achieved *via* routes including histone methylation/demethylation, acetylation[70,71] or the activation of transcription factors such as STAT-6[72]. In a mouse model with gut targeted expression of human 15-LOX-1 exposed to azoxymethane, the number of tumors was lower in

the animals with transgene expression, and 15-LOX-1 expression was lower in samples of tumor tissue compared to normal tissue[73].

An inverse link between 15-LOX-1 expression and secretion of pro-inflammatory cytokines has been indicated in recent years. Gut-targeted expression of 15-LOX-1 has resulted in lower levels of TNFα and inducible nitric oxide synthase (iNOS) in epithelial cells[73]. In human CRC, down-regulation of 15-LOX-1 was associated with increased IL-1β expression[74]. This was further substantiated by a loss of NF-κB (a key inflammatory transcription factor) signaling both in colon cancer cell lines and mouse models when 15-LOX-1 was re-expressed in the gut[73,75,76]. Additionally, there is evidence indicating that 15-LOX-1 expression can inhibit CAC. Chemical inhibition of 15-LOX-1 by PD146176 was shown to cause significant deterioration of intestinal functions in a murine model of experimental colitis[77]. While LA is efficiently oxygenated by 15-LOX-1 to produce 13(S)-HODE, AA can also be metabolized by both 15-LOX-1 and 15-LOX-2 to produce 15(S)-HETE[78]. 15(S)-HETE levels were reported to be significantly lower in the serum of colorectal cancer patients when compared to their levels in control subjects[78].

Thus, activation of acute inflammatory responses, anti-inflammatory and anti-tumorigenic pathways or a neoplastic transformation may occur as a result of the opposing effects of various metabolites formed downstream of the enzymatic action of different LOXs De-regulation of any of these pathways may lead to a loss of homeostasis.

EICOSANOIDS AND THE HALLMARKS OF CANCER

Various types of cancer cells and surrounding stromal cells produce high amounts of pro-inflammatory eicosanoids. These bioactive lipid metabolites can modulate tumor progression through several mechanisms, including directly activating receptors on tumor epithelial cells that help regulate cell proliferation, apoptosis, migration, and invasion, and inducing epithelial cells to secrete growth factors, pro-inflammatory mediators, and angiogenic factors. All of these molecules can facilitate tumor growth, and also support tumor-associated angiogenesis and evasion of the immune system[20].

Proliferation and apoptosis

It is already well documented that tumor growth relies on a deregulated balance between cellular proliferation and cell death. It is not surprising that various eicosanoids that activate/inhibit important signaling pathways in cells can also regulate cellular proliferation and apoptosis in colon cancer cells.

COX-2 pathway in cell proliferation and apoptosis

COX-2 is overexpressed in 50%-80% of all colorectal cancers[79]. At the cellular level, COX-2 overexpression was shown to increase cell-to-matrix adhesion and inhibit apoptosis in human CRC cells[80-82]. Furthermore, in the $APC^{\Delta 716}$ mouse model, the number and size of the polyps were shown to be reduced dramatically when the *COX-2* gene was knocked out[83]. In accordance with this finding, ASA and sulindac have been shown to reduce the number and size of adenomatous colonic polyps in patients with FAP[84]. Additionally, conventional NSAIDs are known to inhibit chemically-induced colon cancer in rodent models by inhibiting COX-2 activity[85]. In the human colon cancer cell line HCT-116, COX-2 was induced through wild-type p53-mediated activation of the Ras/Raf/ERK cascade, which subsequently blocked p53 or genotoxic stress-mediated apoptosis. This anti-apoptotic effect may represent a mechanism for diminishing cellular stress associated with p53 induction[86]. On the other hand, NSAIDs inhibited expression of the anti-apoptotic protein Bcl-X_L, resulting in an altered BAX to Bcl-X_L ratio and enhanced apoptosis[87]. Increased expression of the anti-apoptotic protein Bcl-2 and reduced expression of the pro-apoptotic protein Bim caused by the COX-2-derived eicosanoid PGE_2 have also been reported[21,88].

A considerable amount of crosstalk has been reported between the COX-2 and EGFR pathways. For instance, PGE_2 treatment was shown to significantly increase cellular proliferation and reduce apoptosis in a rodent CAC model[89], and also induce COX-2 expression in intestinal adenomas by activating the MAPK signaling pathway[90]. PGE_2 was shown to induce ERK2 signaling in colon cancer cell lines by stimulating the rapid phosphorylation of EGFR[91]. Inhibition of both EGFR and COX-2 achieved by using a targeted liposome carrying the COX-2 inhibitor celecoxib and a monoclonal antibody against EGFR (Cetuximab) has been shown to additively inhibit the proliferation of colon cancer cell lines expressing both EGFR and COX-2[92].

Roberts *et al*[93] reported that during glucose deprivation, PGE_2 can promote tumor cell survival in the colon by activating the PI3K/AKT pathway, which in turn may up-regulate COX-2 and down-regulate 15-PGDH. Moreover, glucose deprivation was also demonstrated to activate the unfolded protein response (UPR), resulting in elevated expression of the C/EBP-homologous protein (CHOP), which was positively correlated with 15-PGDH expression. These data suggest that stress conditions can regulate PGE_2 as a common and crucial mediator of cell survival during adaptation to the tumor microenvironment.

In the colorectal adenocarcinoma cell line DLD-1, PGE_2 was shown to bind to EP_2, which stimulated tumor growth by activating PI-3K/AKT signaling,

followed by activation of the β-catenin signaling pathway[94]. PGE_2 can also induce cell proliferation in colorectal tumors through the EP_4 receptor by inducing phosphorylation of ERK[95]. Additionally, Park *et al*[96] have proposed that COX-2 inhibition may produce significant anti-tumorigenic effects by blocking stroma-derived PGs. These authors used a co-culture model to evaluate cancer cell-stromal cell relationships and reported that use of an EP_4 antagonist resulted in decreased proliferation in the COX-2 non-expressing LS174T colon adenocarcinoma cell line.

In contrast to PGE_2, 15d-PGJ_2 was shown to induce apoptosis[97] and cell cycle arrest in CRC cells[98] by inhibiting activity of the inflammatory transcription factor NF-κB, reducing the levels of anti-apoptotic genes[97], down-regulating c-Myc expression, and upregulating c-Jun and GADD153[99]. When 15d-PGJ_2 and histone deacetylase (HDAC) inhibitors were added in combination to colon cancer cell lines, they exerted a synergistic effect on caspase-dependent apoptosis, leading to ROS generation, ER stress, decreased expression of anti-apoptotic proteins Bcl-X_L and XIAP, and increased expression of CHOP and DR5 (Death receptor 5, TRAIL-R2). Furthermore, the same effects of this co-treatment were also seen *in vivo*, with an inhibition in tumor growth in a nude mouse xenograft model inoculated with DLD-1 cells[100]. Shin *et al*[101] suggested that the growth inhibition and 15d-PGJ_2-induced apoptosis seen in human and murine CRC cell lines were caused by ROS dependent down-regulation of AKT and p-AKT.

LOX pathways in proliferation and apoptosis

The 5-LOX protein is overexpressed in the early stages of colon cancer, where its expression is significantly correlated with patient age, polyp size, and the presence of intraepithelial neoplasia and villous and tubulovillous adenoma, all of which are considered to be typical markers of transformed adenomatous polyps[102]. Inhibition of 5-LOX with Zileuton was shown to significantly decrease proliferation in a colon cancer cell line and reduce the size of xenografted tumors[57]. LTB_4 demonstrated pro-carcinogenic effects in CRC by activating the ERK pathway[103]. Induction and/or accumulation of COX-2, β-catenin, and Bcl-2, as well as PGE_2 production in non-transformed epithelial linings in the colon have also been reported in the presence of LTB_4[104]. Furthermore, LTD_4, a cysteinyl leukotriene, was reported to inhibit caspase 3, and thereby increase resistance to NSAID-induced cell death[105].

In several different cancer types, COX-2 and 5-LOX signaling can converge to enhance cell proliferation[106]. For example, knock-out of 5-LOX or FLAP was shown to increase the amount of COX-2 metabolites produced by inflammatory cells, indicating that inhibition of one pathway could shunt metabolism of AA towards the other pathway[107-109]. Dual inhibition of 5-LOX and COX-2 may produce additive or synergistic effects on reducing cellular proliferation in colon cancer, as shown by the combination of AA861 (5-LOX inhibitor) and celecoxib[110], the dual COX/5-LOX inhibitor licofelone[111], and the combination of celecoxib and MK886 (5-LOX inhibitor)[112]. Gaining a better understanding of these pathways will have important implications for cancer chemoprevention and treatment[18].

The role of 15-LOX-1 in proliferation and apoptosis of colon cancer was initially considered to be controversial, although well-controlled *in vitro* and *in vivo* studies conducted in the past several years have revealed an unequivocal tumor suppressive role for 15-LOX-1 in CRC[113]. While initial studies indicated an anti-apoptotic role for the enzyme, those investigations were primarily conducted using inhibitors such as NDGA (nordihydroguaiaretic acid), which may have pleiotropic effects in cells[114]. Yoshinaga *et al*[115] reported that 15-LOX-1 over-expression in colon cancer cell lines increased cell proliferation *via* activation of ERK, followed by a decrease in $p21^{(Cip/WAF1)}$ expression. However, numerous subsequent studies have shown that the main product of 15-LOX-1, 13(S)-HODE, can inhibit cell proliferation and induce apoptosis in CRC[116,117]. Moreover, both 15-LOX-1 expression and levels of 13(S)-HODE were reduced in the polyps when compared to paired normal tissues obtained from patients with FAP[67]. Mice express 12/15-LOX, an enzyme that can simultaneously metabolize AA to 12-HETE and LA to 13(S)-HODE, which have opposing effects on tumorigenesis. Therefore a transgenic mouse model was established that can express human 15-LOX-1 specifically in gut epithelial cells[74]. These mice showed a decreased incidence of tumors[73]. Interestingly, an inverse correlation between 15-LOX-1 and COX-2 expression has been proposed to occur during the adenoma to carcinoma sequelae[118], leading to the accumulation of pro-tumorigenic PGs and the loss of apoptotic 13(S)-HODE. It has been suggested that 15-LOX-1-mediated inhibition of NF-κB, which can transcriptionally up-regulate COX-2, leads to a loss of expression of the latter. Epigenetic silencing of 15-LOX-1 in the later stages of progressive CRC may lead to an increase in COX-2 expression, and thus exacerbate the inflammatory milieu[119].

However, focusing on the effects of 15-LOX-1 expression only in epithelial intestinal cells may not provide sufficient information about how its expression contributes to CRC development. Additional knowledge concerning the effects of 15-LOX-1 and its metabolites on tumor-associated stromal cells and endothelial cells is also required to understand the underlying mechanisms of CRC development that lie beyond 15-LOX-1 signaling.

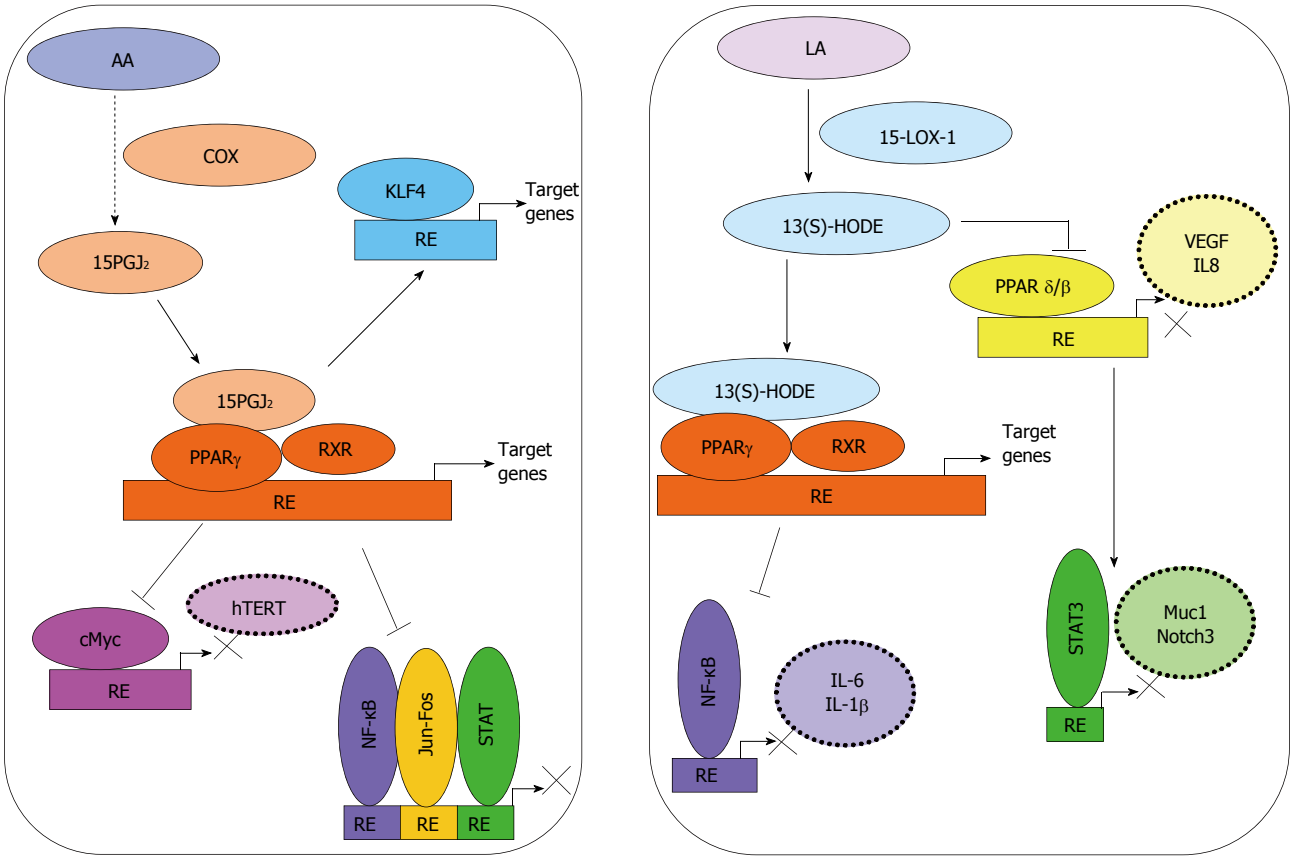

Figure 2 Activation of PPARγ by bioactive lipids can modulate signaling in progressive colorectal cancer. 15-deoxy-delta(12,14)-prostaglandin J2 (15-PGJ2), generated from arachidonic acid (AA) by the enzymatic action of COX-2, acts as a ligand for PPARγ. Co-activators such as RXR activation of tumor suppressive signaling through Kruppel-like factor 4 (KLF4) in colorectal cancer (CRC) have also been reported. Binding to co-repressors may lead to repression of various transcription factors such as nuclear factor kappa B (NF-κB), AP1 (Activator Protein-1, c-Jun and c-Fos), c-Myc or STAT. 13(S)-hydroxyoctadecadienoic acid [13(S)-HODE], generated by oxygenation of linoleic acid (LA) by 15-LOX-1, can act as a ligand for PPARγ and lead to inhibition of NF-κB activity. 13(S)-HODE may also inhibit the transcriptional activities of PPARβ/δ and STAT3, and thereby reduce inflammation and angiogenesis in CRC.

NF-κB and PPAR signaling pathways driven by eicosanoids in CRC

In colon cancer, the activity of NF-κB in intestinal epithelial cells and myeloid cells in the tumor environment plays an essential role in tumor formation[76]. Therefore, one may suggest that specific inactivation of the NF-κB pathway in cancer cells and surrounding myeloid cells may attenuate formation of inflammation-associated tumors[120].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of a nuclear hormone receptor superfamily that includes PPARα, PPARγ, and PPARβ/δ. Each of these receptors can mediate the physiological actions of numerous fatty acids and fatty acid-derived molecules that serve as ligands for these transcription factors (Figure 2). Activated PPARs can also function as transcriptional repressors of NF-κB, STAT-1, and AP-1 signaling[121]. PPARγ is known to be expressed in normal colon tissue, and show reduced expression in colon tumors[122]; however, mutations of PPARγ in CRC are rare[123]. Agonists of PPARα and PPARγ were shown to inhibit DSS-induced colitis and formation of ACF in rats[124]. On the other hand, PPARβ/δ is associated with pro-inflammatory pathways and progression of CRC[121].

15d-PGJ2, a natural agonist of PPARγ, was shown to inhibit the proliferation of HT-29 human colon cancer cells by up-regulating tumor suppressive transcription factor, Kruppel-like factor 4 (Klf-4)[125]. 15d-PGJ2 and rosiglitazone, a synthetic ligand of PPARγ, were found to suppress proliferation of Caco-2 CRC cells by repressing telomerase activity and telomerase reverse transcriptase (hTERT) expression by down-regulating c-Myc and up-regulating Mad1[126].

13(S)-HODE produced in the 15-LOX-1 pathway can act as a ligand for PPARγ[127]. Re-expression of 15-LOX-1 in colon cancer cells was shown to down-regulate PPARδ, and thereby promote induction of endogenous PPARγ target genes related to induction of apoptosis[128]. In support of this finding, over-expression of 15-LOX-1 was associated with decreased proliferation and increased apoptosis, as well as reduced cellular motility, anchorage-independent growth, migration, and cell invasion in colon cancer cells[118]. Moreover, increased 13(S)-HODE-mediated PPARγ activation has been suggested to inhibit activation of NF-κB, which is associated with decreased cell viability[75]. In colitis and CAC[124], 15-LOX-1

activity was also shown to activate PPAR-γ[128,129], which suppressed the expression of key inflammatory genes; most likely by inhibiting NF-κB[130,131].

13-(S)HODE has been shown to suppress PPAR-δ; a receptor that can transcriptionally upregulate IL-6 expression, and thereby promote colitis and CAC[128,132,133]. Very recently, 15-LOX-1-induced inhibition of PPARδ during promotion of CAC was shown to be mediated by suppression of IL-6 expression, STAT3 phosphorylation, and Notch3 expression[134]. Moreover, elevated expression of PPARδ has been implicated in the pathogenesis of CRC[135,136], and a positive correlation between PPARδ expression and the late stages of CRC has also been observed[137]. PGI_2 was shown to activate PPARδ, which may lead to a loss of apoptosis through sequestering of the pro-apoptotic protein BAD by 14-3-3 epsilon and reduced mitochondrial damage[138]. Similarly, stromal PGI_2 generation was claimed to promote cell survival in colonocytes by activating PPARδ[139]. More recently, PPARδ activation was also associated with an increased expression of VEGF and IL-18 in colon cancer cells, through p300 and the PI3K/AKT pathway. Furthermore, hypoxia stimulated PPARδ activation enhanced angiogenesis, macrophage recruitment, and macrophage proliferation in the tumor microenvironment[140].

Metastasis

Although surgery is the most curative approach for CRC, approximately 40% of treated patients eventually show either local recurrence or distant metastases[141,142], primarily to the liver and lungs[143]. Both experimental and clinical studies have shown that daily use of ASA was associated with a reduced risk of metastasis[144], and inhibited the spread of primary tumor cells to other organs post-diagnosis[145]. These findings suggest a role for eicosanoids and eicosanoid-mediated signaling in CRC metastasis.

COX pathway and metastasis

Metastasis is a well-regulated cascade of events that requires the coordinated activation of several factors expressed/released not only by tumor cells, but also by stromal cells. PGE_2 is claimed to promote a more metastatic CRC phenotype[146]. An analysis of sporadic colorectal adenocarcinoma tissue samples revealed a significant relationship between COX-2-derived PGE_2 levels and tumor stage: higher PGE_2 levels were reported in metastatic tumor specimens when compared to tumor specimens without metastases. Thus, it can be concluded that PGE_2 levels may be correlated with tumor aggressiveness, its ability to metastasize, and patient prognosis[147].

Epidemiological, clinical, and animal studies have demonstrated that COX-2 and epidermal growth factor (EGF) signaling pathways coordinate their activities to play key roles in promoting CRC growth and metastasis[148]. For example, EGFR expression was directly correlated with the potential of human CRC cells to metastasize to the liver[149]. Moreover, Buchanan *et al*[150] suggested that in early stage CRC, the initial effects of PGE_2 were mediated by EGFR transactivation and subsequent phosphorylation, which was also responsible for down-stream effects, including cell migration and invasion. In their following reports, the same group showed that PGE_2 activated an EP_4/β-arrestin1/c-Src signaling complex, resulting in EGFR transactivation and downstream Akt signaling, which subsequently stimulated migration of CRC cells *in vitro* as well as their metastatic spread to the liver *in vivo*[151]. Additionally, in the presence of functional EGFR, PGE_2 was shown to transactivate hepatocyte growth factor receptor (c-Met-R), and thereby increase phosphorylation and accumulation of the oncogene β-catenin. This sequence of events induced expression of urokinase-type plasminogen activator receptor (uPAR), resulting in increased CRC cell invasiveness[152]. A significant decrease in liver metastasis with the use of selective EP_4 receptor antagonists has also been reported[153]. In another report, PGE_2 treatment was shown to activate JNK1/2 kinase. This activation was followed by an increase in the levels of migration-related factors uPA and MMP-9, which further promoted cellular motility in the human colon cancer cell line LoVo. However, pretreatment with 17β-estradiol down-regulated uPA and MMP-9 expression *via* deactivation of JNK1/2, and inhibited PGE_2-induced LoVo cell motility. Based on these findings, the authors suggested that the incidence and mortality rates of CRC in women were lower than those in men because estrogen helps protect against development of fatal colon cancer, and thus reduces mortality from this disease[154].

In contrast to PGE_2, PGI_2 is known for its anti-metastatic effects in CRC. PGI_2 analogues have been suggested to protect against metastasis by inhibiting CAM (Cell Adhesion Molecule) -mediated adherence of colon carcinoma to endothelial cells in metastatic target organs[155].

LOX pathway and metastasis

Data concerning the roles of LOX enzymes in colon cancer migration and invasion have recently been reported[156]. In one study, decreased levels of the selective LOX inhibitor, NDGA, were found in mobile human colon cancer cells; this was partly explained by inhibition of MMP-2 and MMP-9[157]. Loss of 15-LOX-1 expression was found in the lymph node and liver metastases of pancreatic cancer[158], and 15-LOX-1 re-expression in CRC cell lines inhibited their invasiveness, motility and migration[117]. More recently, Wu *et al*[159] showed that 15-LOX-1 re-expression in HCT116, HT29, and LoVo colon cancer cells inhibited cell survival, as well as angiogenesis, cancer cell migration, and invasion.

Angiogenesis

For tumors to grow and metastasize, they must generate their own blood supply; a process defined as neo-angiogenesis. Many cells in the tumor microenvironment, including tumor epithelial cells, stromal cells, and immune cells, secrete various pro-angiogenic factors needed for proliferation, migration, capillary tube formation, and recruitment of endothelial cells[160]. Numerous *in vitro* and *in vivo* studies have shown that eicosanoids can modulate angiogenesis at different levels[20].

VEGF is a major regulator of angiogenesis, and its expression is up-regulated in response to multiple micro-environmental "stress" factors such as hypoxia, acidosis, and starvation; which are all related to poor blood supply. In tumors, hypoxia can lead to stabilization of transcription factor HIF-1α, which activates genes which have the hypoxia-responsive element (HRE) in their promoter region, such as *VEGF*. VEGF exerts its effects on target cells through tyrosine kinase receptors, including VEGF receptors 1 (VEGFR1, Flt1) and 2 (VEGFR-2, Flk-1/KDR)[161]. Ligand binding induces receptor dimerization and activation of downstream signaling pathways including the MAPK family, PI3K/AKT and protein kinase C (PKC)[161]. Besides hypoxia, other factors that have been shown to stimulate VEGF expression include ROS[162], growth factors[163], cytokines[164], and various lipid mediators such as PGE_2[165-168].

COX pathway in angiogenesis

PGE_2 stimulation has been shown to induce HIF-1α stabilization[163] and VEGF expression *in vitro*[169]. Additionally, VEGF and COX-2 expression and tumor angiogenesis were shown to be highly correlated in samples of colon cancer tissue[147,170]. Through its receptor EP_2, PGE_2 was shown to stimulate the nuclear translocation of β-catenin[94], whereby it activated T cell factor 4 (TCF-4) and HIF-1α to trigger cell survival, proliferation, and angiogenesis in colon cancer[171,172]. Homozygous knock-out of EP_2 completely abrogated induction of VEGF in the intestinal polyp stroma of $APC^{\Delta 716}$ mice. It also decreased the number and size of intestinal polyps, showing that PGE_2-directed induction of VEGF was an important factor for tumor growth[173]. Moreover, PGE_2 was shown to induce expression and release of the pro-angiogenic chemokine CXCL1 in CRC, which in turn stimulated microvascular endothelial cell migration and tube formation both *in vitro* and *in vivo*[174]. Hypoxia was shown to induce EP_1 expression in colon cancer cells, while inactivation of EP_1 inhibited PGE_2-dependent and hypoxia-inducible expression of angiopoietin-like protein 4 (ANGPTL4), whose lipid metabolizing functions are exerted *via* inhibition of lipoprotein lipase (LPL)[175].

In addition to inducing several angiogenic factors in epithelial cells, PG signaling in surrounding stromal cells also supports angiogenesis in colon cancer. For example, PGE_2 and TXA_2 were reported to regulate the adhesion and spreading of human umbilical vein endothelial cells (HUVEC) through cAMP-dependent activation of protein kinase A (PKA) and cAMP- and PKA-dependent activation of Rac, respectively[176]. Besides VEGF, PGE_2 may also mediate the angiogenic effects of basic fibroblast growth factor (bFGF) by up-regulating expression of C-X-C chemokine receptor type 4 (CXCR4) in human microvascular endothelial cells (HMECs), and enhancing cellular response to stromal-derived factor 1 (SDF-1), a unique ligand for CXCR4[177]. TXA_2 has been shown to enhance endothelial cell migration and angiogenesis[178]. An increase in TXA_2 levels, as a result of overexpression of TXA_2 synthase in C-26 colon adenocarcinoma cells allografted to BALB/c mice, was reported to stimulate accelerated tumor growth and tumor-associated angiogenesis[179].

The process of angiogenesis may require not only crosstalk between tumor epithelial and endothelial cells, but also the involvement of immune cells that produce pro-angiogenic factors. PGE_2 has been shown to induce mast cells to release VEGF and the chemokine CCL2[180,181], which can induce tumor-associated angiogenesis by directly recruiting CCR2 expressing endothelial cells and inducing VEGF release from macrophages[26]. Contrary to the pro-angiogenic roles described above, PGE_2, through its receptor EP_2, was shown to inhibit secretion of VEGF in Caco-2 colon cancer cells exposed to hyperosmotic stress[182]. Additionally, 15d-PGJ_2 was shown to down-regulate COX-2 and VEGF expression in colon carcinoma cells by inhibiting the transcription factor AP-1[183]. In an *in vivo* study in which PGI_2 synthase was retrovirally transduced into C-26 colon adenocarcinoma cells and subsequently grafted to syngeneic BALB/c mice, the increased production of PGI_2 resulted in slower tumor growth and a decreased amount of vasculature[179].

When viewed in total, these findings suggest that relative levels of pro- and anti-angiogenic prostanoids in the tumor microenvironment might be strong determinants of the degree of angiogenesis that occurs in colorectal tumors.

LOX pathway in angiogenesis

A growing body of evidence indicates that LOX-catalyzed products (LTs and HETEs) also exhibit important biological effects on the angiogenic process in colon tumors. Ye *et al*[184] implicated 5-LOX in the promotion of colon cancer growth by nicotine through up-regulation of VEGF, MMP-2, and MMP-9, resulting in stimulation of angiogenesis in the colon. The same group also reported that cigarette smoke extract indirectly stimulated endothelial cell proliferation, a biological phenomenon that may enhance neo-angiogenesis[185,186]. CysLT1R antagonists were shown to impair angiogenesis in colon cancer xenografts[187], while LTB_4 was reported to induce neutrophil-mediated

vascular permeability[188]. Additionally, LTB_4 was shown to enhance hypoxia-induced microvascular alterations *in vivo*[189]. Expression of the LTB_4 receptor BLT2 was found to be highly inducible by VEGF, suggesting interplay among VEGF, BLT2, and BLT2 ligands during vascular angiogenesis[190]. Similarly, LTC_4 and LTD_4 also promoted angiogenesis *via* receptor-mediated interactions[191]. Moreover, reduced vascular permeability was observed in LTC_4 synthase knock-out mice which had impaired synthesis of cysteinyl LTs[192].

Very few reports have addressed the role of 15-LOX-1 in neo-angiogenesis in colorectal cancer. Recently, our group and others have shown that re-expression of 15-LOX-1 in colon cancer cell lines could reduce the expression and secretion of VEGF-A, and that treatment of HUVECs with conditioned medium from colon cancer cell lines ectopically expressing 15-LOX-1 resulted in reduced tube formation[159,193]. However, the signaling mechanism that medicates this angiostatic effect has not yet been reported.

Therefore, as with the prostanoids, it is likely that different bioactive lipids produced by the LOX pathway may have contrasting effects on angiogenesis, and their ultimate functional effects may be decided by the balance between pro- and anti-angiogenic products.

EICOSANOIDS IN THE RESOLUTION OF INFLAMMATION

The resolution of acute inflammation, rather than being a passive process of diluting out pro-inflammatory mediators, was shown to be actively conducted by several different bioactive lipid mediators[194]. The timely resolution of inflammation prevents the development of chronic inflammation and fibrosis, and enables an organism regain a state of homeostasis[194].

The primary drivers of resolution include cessation of neutrophil infiltration and the nonphlogistic recruitment of macrophages to clear debris at the site of inflammation[194]. Lipoxins (Lx) were the first bioactive lipids to be identified as mediators of these processes. Lx's can be synthesized from AA in neutrophils through the enzymatic action of 5-LOX, and LTA_4 can be converted to LXA_4 and LXB_4 by 12-LOX in platelets upon the latter's adherence to neutrophils[195] (Figure 1). Additionally, AA can be metabolized by 15-LOX-1; after which, the oxygenated product can be converted to an epoxytetraene, and then to LXA_4 or LXB_4 by the action of hydrolases. ASA stimulates acetylation of COX-2, and thus shifts the activity of that enzyme from production of pro-inflammatory prostanoids to production 15(R)-HETE, which is subsequently metabolized by 5-LOX to 15-epi-Lx or aspirin triggered lipoxins (ATLs)[18]. Many of these bioactive lipids act through G-protein coupled receptors such as the lipoxin receptor/formyl peptide receptor (ALX/FPR2), which binds to LXA_4 and ATLs[18]. Although most autacoids involved in resolution are known to be synthesized in a transcellular manner involving at least two cell types, a recent study indicates that lipoxins may also be generated from a single immune cell[196].

LXA_4 expression or administration of LXA_4 analogs has been shown to reduce DSS-induced colitis[197]. Inflammatory stimuli in intestinal epithelial cells have been shown to initiate a feedback reaction which up-regulates expression of the LXA_4 receptor in intestinal epithelial cells[198]. Additionally, co-culture of Caco-2 cells with macrophages, where the cells were also treated with LXA_4, resulted in decreased secretion of pro-inflammatory cytokines[199], most likely due to inhibition of NF-κB. In a recent study which used colonic biopsies obtained from patients experiencing a remission of ulcerative colitis, significantly increased levels of LXA_4, along with enhanced expression of FPR2/ALX receptor mRNA and increased levels of macrophage infiltration were observed, suggesting that LXA_4 levels may play an important role in restoring mucosal homeostasis[200]. FPR2 expression was also shown to be increased in the colons of patients with Crohn's disease; again indicating that signaling through lipoxin was enhanced in the same inflammatory environments that were most likely to have enhanced clearance of debris or bacteria by macrophages[201].

Resolvins (Rvs) are derived from the n-3 fatty acids EPA (E-series Rvs) and DHA (D-series Rvs), and formed by the concerted actions of acetylated COX-2, 5-LOX or 15-LOX[18] (Figure 1). Rvs have demonstrated potency at very low concentrations when administered orally or intravenously[18], and are known to signal through *ChemR23* and chemokine-like receptor 1 (CMKLR)[18]. RvD1 and RvD2 in the CACs of mice are known to be chemopreventive, and help reduce tumor growth[202]. RvE1 was reported to induce the clearance of neutrophils into the lumen of the gastrointestinal tract[203]. Moreover, RvE1 was shown to inhibit phosphorylation and activation of p65 NF-κB in the distal colons of mice in a DSS-colitis mouse model[199], suggesting its roles in both pro-resolution and anti-inflammatory pathways. Interestingly, the enzyme intestinal alkaline phosphatase (ALP1), a marker of differentiation, was shown to be induced in epithelial cells in the presence of RvE1, and also have a role in protecting against colitis[204]. Many of these potent bioactive molecules are currently being studied in large-scale clinical trials[205].

Mareisins (MaR) are generated in macrophages from DHA through the action of macrophage 12-LOX[202]. Intermediates formed during the conversion of DHA to MaR1 were shown to inhibit formation of LTB_4 and oxygenation of AA by 12-LOX, but enhance conversion of M1 inflammatory macrophages to the M2 phenotype[202]. Another recently identified intermediate is 13,14-dihydroxydocosahexaenoic acid (13,14-diHDHA or MaR2), which is synthesized when macrophages are co-incubated with 12-LOX and soluble epoxide hydrolase (sEH). This compound has been shown to reduce neutrophil migration and

enhance macrophage phagocytosis at nanogram concentrations[202]. MaR1 was recently used in a DSS and TNBS-induced mouse model of colitis. In that model, the disease activity index, amount of body weight loss, and tissue damage in the colon were all reduced. Additionally, there were significant decreases in the levels of inflammatory cytokines; most likely resulting from inhibition of the NF-κB pathway[206]. Moreover, in the same study, reduced migration of neutrophils, reduced ROS production, and reduced levels of inflammatory cytokines in LPS-stimulated bone marrow-derived macrophage cultures incubated with MaR1 were also reported[206].

CONCLUSION

There is no doubt that eicosanoids are an important family of immunoregulatory bioactive lipids with involvement in both the promotion and prevention of colon cancer. During inflammation, many of these autacoids act antagonistically or synergistically, and often in a temporal manner with the aid of different cell types in order to induce homeostasis. Many of these bioactive lipids are also essential for various cellular functions. Despite its importance, very few therapeutic agents are available that can modulate the aberrant production of these molecules specifically in the context of colorectal or other cancers. ASA is undoubtedly one of the best known drugs that interferes with the COX pathway; however, ASA needs to be consumed long term (at least 5 years) to provide any protective effect against cancer. Furthermore, use of ASA is associated with significant bleeding events, and is therefore not suitable for all patients. COX-2 inhibitors that specifically target the inflammatory arm of the COX metabolic pathway are approved primarily for pain relief rather than for cancer chemotherapy, and are also associated with significant cardiovascular side effects. On the other hand, CysLTR antagonists that were originally designed for asthma have also not demonstrated high levels of efficacy[207]. Because inhibition of one pathway leads to activation of another due to the shunting of substrates, combined COX/LOX inhibitors have proved to be more effective than signal pathway inhibitors, and should be further studied in the context of CRC.

The scientific community needs to develop drugs that are specifically effective in cancer, and perhaps the most promising candidates include newly discovered resolution mediators such as lipoxins, resolvins, and mareisins. The results of early studies have indicated that these mediators are effective at very low concentrations, and therefore may be viable chemopreventive/therapeutic agents for use in CRC. It is also interesting to note that COX-2 and 5-LOX, that are associated with pro-carcinogenic events, and 15LOX-1, which is associated with anti-carcinogenic events in CRC, rarely show any mutations. Deregulation in their activity results from their overexpression, enhanced enzymatic activity or epigenetic silencing. Therefore, one may envisage the design and development of chromatin modifiers capable of reducing the expression of pro-inflammatory enzymes such as COX-2 and 5-LOX, while enhancing the expression of the anti-inflammatory enzymes such as 15-LOX-1.

There is no dearth of information in the literature highlighting the importance of eicosanoids in cancer. Delving into the details of how eicosanoids function in both tumor and stromal cells will be essential for understanding the pathways involved. Such knowledge will aid in the design of novel cancer therapies.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Colorectal cancer: Metastases to a single organ

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Abstract

Colorectal cancer (CRC) is a common malignancy worldwide. In CRC patients, metastases are the main cause of cancer-related mortality. In a group of metastatic CRC patients, the metastases are limited to a single site (solitary organ); the liver and lungs are the most commonly involved sites. When metastatic disease is limited to the liver and/or lungs, the resectability of the metastatic lesions will dictate the management approach and the outcome. Less commonly, the site of solitary organ CRC metastasis is the peritoneum. In these patients, cytoreduction followed by hyperthermic intraperitoneal chemotherapy may improve the outcome. Rarely, CRC involves other organs, such as the brain, bone, adrenals and spleen, as the only site of metastatic disease. There are limited data to guide clinical practice in these cases. Here, we have reviewed the disease characteristics, management approaches and prognosis based on the metastatic disease site in patients with CRC with metastases to a single organ.

Key words: Colorectal cancer; Metastasis; Prognosis; Disease management; Liver metastasis; Lung metastasis; Brain metastasis; Bone metastasis; Peritoneal metastasis

Core tip: Colorectal cancer (CRC) is a common malignancy. In CRC patients, metastases are the main cause of cancer-related mortality. Cancer spread can sometimes be limited to a single organ, representing a malignancy with a distinct biological profile and clinical characteristics. In CRC patients with single site metastases, the resectability of the metastases and the site of metastatic disease affect the clinical characteristics, the optimal management approach and the prognosis.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide[1] and continues to be one of the leading causes of cancer-related death globally[2]. In CRC patients, similar to those with other malignancies, metastases are the main cause of cancer-related mortality. Distant metastatic disease is present in approximately 25% of patients at initial diagnosis, and half of CRC patients will develop metastatic disease[3]. Most patients with metastatic CRC have incurable disease. In this group of patients, median survival has improved from less than 10 mo with best supportive care to 14 mo with fluoropyrimidine treatment[4,5] and to more than 2 years with a combination of various cytotoxic[6,7] and biologic agents[8,9].

The concept of oligometastatic cancer was first proposed two decades ago[10]. Cancer spread can sometimes be limited to a single organ, representing a malignancy with a peculiar biological profile and clinical characteristics. In this group of patients, the prognosis differs significantly: some patients with resectable metastases can be offered potentially curative treatments, and occasionally, chemotherapy can be used to render the metastases resectable[11], but the prognosis in others remains grim. Numerous factors are involved in this difference in prognosis. Here, we have reviewed the literature to clarify the prognosis of CRC patients with metastases to a single site (solitary organ) and the prognostic role of the metastatic disease site.

LIVER

The liver is the most common site of metastasis from CRC; this is thought to be due to the venous drainage of the colon and rectum. Approximately 50% of CRC patients will develop liver metastasis during the course of the disease[12]. In patients with metastatic CRC, the liver is the sole organ with metastases in approximately one-third of patients[13]. In a retrospective analysis of 780 patients with CRC and liver-only metastases (including both resectable and non-resectable cases), the median overall survival (mOS) was reported as 22.8 mo[14]. Depending on the resectability of the metastases, patients with liver metastases from CRC have different prognoses.

RESECTABLE LIVER METASTASIS

Approximately 20% of patients with hepatic metastases present with resectable disease at diagnosis[15]. According to one consensus statement, contraindications to resection include the following: unresectable extrahepatic disease, more than 70% liver involvement, liver failure, and being surgically unfit[16]. Nevertheless, the selection criteria for hepatic resection are evolving and are beyond the scope of this review. In patients who undergo resection of liver metastases, a 5-year survival of 25% to 58%[17-25] and a 10-year survival of 17% to 28%[17,19-21,26] have been reported, and one study has reported a 20-year survival of 17%[20] (Table 1). A systematic review of the published data showed a 5-year survival of approximately 30%, with the majority of these patients being disease-free[27]. Studies have shown that a significant number of 5-year survivors progress to cancer-related death[21,28]. However, it appears that patients who survive 10 years are cured[21,29]. In patients with resectable liver metastases, perioperative chemotherapy with the FOLFOX regimen has been shown to improve progression-free survival[30]. However, this approach has not been shown to improve overall survival[31].

Table 1 Hepatic metastasectomy: Large retrospective studies

Ref.	Patients, *n*	Median overall survival (mo)	5-yr survival	10-yr survival	20-yr survival
Rees *et al*[19], 2008	929		36%	23%	
Choti *et al*[17], 2002	226	46	40%	26%	
Fong *et al*[22], 1999	1001	42	37%	22%	
Nordlinger *et al*[18], 1996	1568		28%		
Scheele *et al*[20], 1995	434	40	33%	20%	17%

NON-RESECTABLE LIVER METASTASIS

The majority of patients with liver metastases from primary CRC have non-resectable disease[32-35]. Historical data show that without treatment, these patients have a poor prognosis[36,37]. Retrospective studies of this population suggest that the amount of liver replaced by the tumor is the most significant indicator of outcome[38-42] (Table 2). In a large study by Stangl *et al*[39] that included 484 patients, 189 who did not have extrahepatic metastases had a median survival of 9.6 mo. In this group, patients with a lower volume of liver replaced by tumor and grade 1-2 (primary) disease with no extrahepatic and no mesenteric lymph node involvement had the highest median survival (21.3 mo; range, 5-68 mo; 95%CI: 15.6-34.1). According to these studies, the median survival of patients with CRC metastases confined to the liver with a low disease volume is in the range of 11 to 18 mo; with a higher disease volume, the range is 6 to 8 mo. These studies predate the currently available systemic treatments. Nevertheless, even with the improvements in the survival of patients with metastatic CRC using current chemotherapy regimens,

Table 2 Retrospective studies of patients with unresected liver metastases

Ref.	Patients without extrahepatic metastases, *n*	The extent of liver involvement	Median overall survival (mo)	Treatments
Yamamura *et al*[41], 1997	67	Metastases in one lobe	13	Chemotherapy
(*n* = 73)		Less than four metastases in both lobes	12	(chemotherapy did not significantly
		More than five metastases in both lobes	6	affect survival in multivariate analysis)
Stangl *et al*[39], 1994	189	⩽ 25%	11.1	No treatment
		> 25%	6.3	
Chang *et al*[38], 1989	49		15.1	Floxuridine
(*n* = 67)		< 25%	23.8	(hepatic arterial or intravenously)
		25%-75%	14.8	
		> 75%	7.3	
Arnaud *et al*[40], 1984	(not specified)	One lobe	17	NA
(*n* = 56)		Both lobes	8.23	
Johnson *et al*[42], 1981	51	Solitary liver metastasis (*n* = 12)	18	NA
		Multiple metastases in one lobe (*n* = 6)	7	
		Multiple metastases in both lobes (*n* = 33)	8	

mOS: Median overall survival; NA: Not available.

patients with non-resectable liver metastases have a low 5-year survival rate[43].

In some patients, hepatic metastases that are initially deemed non-resectable can be resected after neoadjuvant chemotherapy[11,44,45]. Similar 5-year survival rates have been reported in this group compared to patients who initially had resectable disease[46]. In some patients with non-resectable liver lesions, radiofrequency ablation is an option that may provide tumor control. In this group of patients, a median survival of 36-59 mo has been reported with local ablation[47-50]. Selective intraoperative radiotherapy (SIRT) is another liver-directed treatment strategy that has been shown to control the progression of metastatic colon cancer within the liver. This involves injecting yttrium-labeled microspheres into the liver *via* the hepatic artery[51]. Randomized phase Ⅲ trials that evaluated the addition of SIRT to systemic chemotherapy as part of the treatment of metastatic CRC limited to the liver are underway[52-54]. The SIRFLOX study results were presented in the recent Annual Meeting of American Society of Clinical Oncology. In this study addition of SIRT to standard chemotherapy did not improve overall progression free survival but improved liver progression free survival. Overall survival results from this study are not available yet[52].

LUNG

The lungs are the second most common site of distant metastases from CRC[55-57]. Previous studies have shown that 10%-15% of patients with CRC develop lung metastases during the course of the disease[58,59]. Compared to colon cancer, patients with rectal cancer are at a higher risk of synchronous and metachronous lung metastases[57-59]. This is believed to be due to direct spread of rectal cancer into systemic circulation through the hemorrhoidal veins[60]. Isolated lung metastases are thought to be less common: in two retrospective studies, 2.8%[58] and 7.4%[61] of patients were reported to have isolated lung metastases. In the multivariate analysis of 5654 CRC patients, those with isolated lung metastases seemed to have a significantly better prognosis compared to those who had another metastatic location in addition to the lungs[58]. One retrospective study showed a significantly better prognosis for patients with lung-only disease compared to those with a single organ metastasis to another organ[14].

RESECTABLE LUNG METASTASIS

In the absence of randomized prospective studies and based on data from retrospective series, it is widely accepted that surgery should be considered for the management of resectable pulmonary metastases from CRC[62-64]. Based on the literature, the suitability criteria for the resection of pulmonary metastases include the following: control of the primary tumor; possible complete resection; and adequate pulmonary reserve to tolerate the planned resection[65]. Various series have shown mOS of 36.2 mo to 49 mo, 5-year survival rates of 32% to 68%[66-77] and 10-year survival rates of 11% to 34%[75,78-80] in patients with CRC undergoing lung metastasectomy (Table 3).

A meta-analysis of the published data has suggested that in this group of patients, the absence of thoracic node involvement, prolonged disease-free interval (between primary tumor and metastatic spread), normal pre-thoracotomy carcinoembryonic antigen (CEA), and a single pulmonary lesion are associated with prolonged survival[63]; of these criteria, the last three have also been shown to be of prognostic value in a separate analysis[64].

NON-RESECTABLE LUNG METASTASIS

In the majority of patients with CRC and pulmonary

Table 3 Retrospective studies including ⩾ 100 patients with resectable lung metastases from colorectal cancer

Ref.	Year	*n*	median survival (mo)	5-yr survival rate (%)
Borasio *et al*[66]	2011	137	36.2	55
Hwang *et al*[67]	2010	125	37	48
Riquet *et al*[68]	2010	127	45	41
Watanabe *et al*[69]	2009	113	NA	68
Welter *et al*[70]	2007	169	47.2	39
Yedibela *et al*[71]	2006	153	43	37
Inoue *et al*[72]	2004	128	49	45
Kanemitsu *et al*[73]	2004	313	38	38
Pfannschmidt *et al*[74]	2003	167	40	32
Saito *et al*[75]	2002	165	NA	40
Zink *et al*[76]	2001	110	41	32

NA: Not available.

involvement, the lung metastases are not resectable. In a prospective study of 70 patients with CRC and isolated unresectable lung metastases who were treated with chemotherapy[81], the mOS was 19 mo (95%CI: 12.6-25.4 mo, range: 5-44 mo), with a 2-year OS rate of 38.8%. The first response assessment seemed to be a prognostic factor, with a mOS of 27 mo (95%CI: 23.4-30.6 mo) for patients with a partial response compared with 16 mo (95%CI: 8.3-23.7 mo) and 8 mo (95%CI: 5.2-10.8 mo) in patients with stable disease and disease progression, respectively ($P < 0.01$). Notably, the lung metastases in a small proportion of patients (5.7%) in this study became resectable with chemotherapy[81]. In a retrospective study of patients with non-resected lung metastases who underwent palliative chemotherapy, Mitry *et al*[58] reported 3-year survival rates of 14.4 and 15.3% for metachronous and synchronous lung metastases, respectively; the 5-year survival rates were reported to be 0 and 8.4%, respectively, for the same cohorts.

PERITONEUM

In retrospective studies of CRC patients, the rate of peritoneal metastases has been reported to be between 4% and 13%[82-85], with the peritoneum as the only site of metastatic disease in approximately 4% of patients[82,84]. The risk factors for peritoneal involvement include right-sided tumor, advanced T-stage, advanced N-stage, poor differentiation grade, mucinous adenocarcinoma and younger age at diagnosis[86]. Historically, peritoneal carcinomatosis from CRC has been associated with poor prognosis with a median survival of 6-8 mo[83,87]. Intraperitoneal chemotherapy has been suggested to improve outcomes in these patients. In a retrospective analysis of 523 patients with CRC and peritoneal involvement as the sole metastatic site who underwent cytoreductive surgery and intraperitoneal chemotherapy, the overall 1-year, 3-year, and 5-year survival rates were 81%, 41%, and 27%, respectively. In that study, the median survival was 30.1 mo[88].

A randomized trial by Verwaal *et al*[89] showed that in patients with peritoneal metastases of CRC or positive cytology of ascites, in the absence of other distant metastases, cytoreduction followed by hyperthermic intraperitoneal chemotherapy (HIPEC) offers a statistically significant advantage in terms of survival (median disease-specific survival of 22.2 mo in the HIPEC arm *vs* 12.6 mo in the standard arm) and progression-free survival (12.6 mo in the HIPEC arm *vs* 7.7 mo in the standard arm). Importantly, in the standard arm of this study, patients received chemotherapy with fluorouracil and leucovorin. In a case-control study, including 48 cases in each arm, patients with resectable peritoneal metastases had a median survival of 24 mo with systemic chemotherapy; in the cytoreduction plus HIPEC group, the median survival reached 62.7 mo, with a 5-year survival rate of 51%[90]. In another study of 67 cases and 38 controls, the mOS was significantly prolonged in the HIPEC group (34.7 mo *vs* 16.8 mo, $P < 0.001$)[91]. In both of the latter studies, both arms received modern systemic chemotherapy regimens[90,91]. This is especially important because in patients with colorectal peritoneal carcinomatosis, modern systemic therapies might be associated with improved outcome (in patients treated systemically alone or with cytoreductive surgery combined with perioperative intraperitoneal chemotherapy)[92].

It appears that in patients with peritoneal metastases from CRC, cytoreductive surgery and intraperitoneal chemotherapy may improve the outcomes, but there is still insufficient evidence in this area[93].

Single organ metastases from CRC are less common in other sites, and below we have reviewed some of these scenarios.

BRAIN

Compared to liver and lung metastases, cerebral metastases from CRC are uncommon. The incidence of brain metastases in patients with CRC has been reported to be between 0.3% and 6% in different series[94-97]. The incidence might be increasing with the recent developments in the treatment of CRC[98]. In patients with brain metastases due to CRC, the primary tumor is more frequently located in the distal colon and rectum rather than in the proximal colon[99,100]. Brain metastases usually occur later in the course of the disease, and most patients already have metastases in other organs, especially the liver and lung, by the time the brain metastases are diagnosed. The prognosis remains dismal, and the median survival of patients with brain metastases from CRC has been reported to be between 3 and 6 mo[99,101-104], which seems to be worse than the median survival of patients with brain metastasis due to other malignancies[102]. In 2%-10% of patients with brain metastasis from CRC, the brain

is the only site of metastatic disease[99,105,106]. In these patients, prognostic factors include age, performance status and a controlled primary tumor[107].

The management approach in patients with brain metastases from solid tumors (including CRC) depends on multiple factors, including the following: patient's performance status, the status of the primary cancer, number/location of the brain lesions, and the presence of leptomeningeal disease[108]. Local treatments for brain metastases include surgery, whole brain radiotherapy (WBRT) and stereotactic radiosurgery[109]. Aggressive local treatments (surgical or radiosurgery in addition to WBRT) improve the outcomes in patients with a good performance status and a limited number of brain metastases[110,111]. Retrospective studies have shown that in selected patients with brain metastases from CRC, the mOS may improve (up to 12-15 mo) with aggressive local treatment of the brain lesions[100,101,106,112-116].

BONE

The incidence of bone metastasis from CRC has been reported to be 10.7% to 23.7% in autopsy series, with signet-ring cell pathology showing a high incidence of bony metastases[117]. One retrospective study using bone scans and plain radiography showed that the incidence of skeletal metastasis in patients with CRC was 6.6%; in this study, 83.1% of the patients had other organ metastases, and 16.9% were deemed to have bony metastases only[118]. One study used positron emission tomography and/or CT scans in 252 patients with a diagnosis of CRC and found the incidence of bone metastasis to be 5.5%, with a median time from diagnosis to the detection of bone metastasis of 21 mo; in this study, none of the patients had bone-only metastatic disease[119]. Different series have shown that the median survival of patients with bone metastases is between 5 to 7 mo after detecting the bone metastases[120,121]. There are only a few case reports of patients with solitary bone metastasis from CRC, with a wide range of prognoses[122-129]. In some of these cases, the use of radiotherapy[123] or surgical resection[128,129] of the bone metastases has been reported to achieve favorable results.

OTHER ORGANS

There are only a few case reports of CRC with isolated metastases to the adrenal glands[130-132] and the spleen[133]. Surgical resection of the metastatic lesion may play a role in such cases.

DISCUSSION

In patients with CRC and single-site metastatic disease, the site of the metastatic disease affects both the treatment approach and the prognosis. In patients with oligometastatic disease limited to the liver and/or the lung (and maybe the peritoneum), the most essential factor that affects the prognosis is the resectability of the metastatic lesion(s). Favorable prognosis in these patients with resectable disease might be due to several factors: in general, such patients have a lower metastatic burden and are in the earlier phases of the disease compared with CRC patients with unresectable metastases, and their performance status is also generally better as they are well enough to tolerate an operation. These confounding factors make it difficult to attribute the improved survival to only one variable; nonetheless, it is likely that the favorable biology of the primary disease plays a role in this complex picture.

In patients with non-resectable oligometastatic disease, those with metastatic disease to the liver or lung have a better prognosis than those with metastases to the peritoneum, brain or bone[14]. The relationship between patterns of metastases from tumors and different prognoses can be explained by the "seed and soil" hypothesis, which was first formulated by Paget[134]. According to this hypothesis, the cancer cells (the seed) must find a suitable microenvironment in the target organ (the soil) for the metastasis to occur. Metastasis is a highly complex process that requires adaptations in the cancer cells as well as cross-talk between the cancer cells and the microenvironment both in the primary tumor as well as in the target organ[135-137]. Previous studies have shown that the involvement of specific organs depends on cancer cell gene expression[138-140]. It is conceivable that such differences in the metastasizing cancer cells not only lead to the involvement of various organs but also have an impact on the outcome of the disease. Other factors may also affect the prognosis of patients with different metastatic sites. One is the effect of the target organ on the cancer cells. Studies have shown that cancer cell gene expression, behavior and response to treatment are affected by the target organ microenvironment[141-144].

In patients with metastatic cancer, including patients with single-site metastatic disease, there are several other factors that may also affect survival, including the involvement of vital organs, which plays an important role in the case of brain metastases. The metastatic burden and the disease volume are also important. In conclusion, in patients with CRC and single-site metastasis, the resectability of the metastases and the metastatic disease site affect the clinical characteristics, the optimal management approach and the prognosis.

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TOPIC HIGHLIGHT

2015 Advances in Colorectal Cancer

Pathophysiological mechanisms of death resistance in colorectal carcinoma

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Abstract

Colon cancers develop adaptive mechanisms to survive under extreme conditions and display hallmarks of unlimited proliferation and resistance to cell death. The deregulation of cell death is a key factor that contributes to chemoresistance in tumors. In a physiological context, balance between cell proliferation and death, and protection against cell damage are fundamental processes for maintaining gut epithelial homeostasis. The mechanisms underlying anti-death cytoprotection and tumor resistance often bear common pathways, and although distinguishing them would be a challenge, it would also provide an opportunity to develop advanced anti-cancer therapeutics. This review will outline cell death pathways (*i.e.*, apoptosis, necrosis, and necroptosis), and discuss cytoprotective strategies in normal intestinal epithelium and death resistance mechanisms of colon tumor. In colorectal cancers, the intracellular mechanisms of death resistance include the direct alteration of apoptotic and necroptotic machinery and the upstream events modulating death effectors such as tumor suppressor gene inactivation and pro-survival signaling pathways. The autocrine, paracrine and exogenous factors within a tumor microenvironment can also instigate resistance against apoptotic and necroptotic cell death in colon cancers through changes in receptor signaling or transporter uptake. The roles of cyclooxygenase-2/prostaglandin E2, growth factors, glucose, and bacterial lipopolysaccharides in colorectal cancer will be highlighted. Targeting anti-death pathways in the colon cancer tissue might be a promising approach outside of anti-proliferation and anti-angiogenesis strategies for developing novel drugs to treat refractory tumors.

Key words: Colon cancer; Tumorigenesis; Anti-apoptosis; Chemoresistance; Anti-necroptosis; Cytoprotection

Core tip: The mechanisms underlying anti-death cytoprotection and tumor resistance bear common pathways, and although distinguishing them would be a challenge, it would also provide an opportunity to develop advanced anti-cancer therapeutics. Auto-

crine, paracrine and exogenous factors within a tumor microenvironment may instigate apoptotic and necroptotic resistance in colon cancers. The roles of cyclooxygenase-2/prostaglandin E2, growth factors, glucose, and bacterial lipopolysaccharide will be highlighted. Targeting death resistance pathways in colon cancer tissue might be a promising approach outside of anti-proliferation and anti-angiogenesis strategies for developing novel drugs to treat refractory tumors.

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INTRODUCTION

Colorectal carcinomas of epithelial origin are characterized by unlimited cell replication, death resistance, and metastasis[1]. In comparison to normal epithelial cells, cancer cells acquire the ability to avoid physiological cell turnover and exhibit an imbalance between renewal and demise, resulting in rapid expansion of tumor mass. It is believed that malignant cells develop adaptive mechanisms for surviving under the extreme conditions of tumor microenvironment, such as restricted oxygen supply and nutrient deprivation. The reprogramming of cancer cells not only contributes to their ability to hyperproliferate but also confers resistance to cell death against endogenous stress and exogenously applied cytotoxic drugs[2]. This review will outline pathways of cell death and discuss how cancer cells manipulate cytoprotective mechanisms to evade it.

MODES OF CELL DEATH

Various types of cell death, *i.e.,* apoptosis, necrosis, and necroptosis, was found in cancer tissues under metabolic stress or cytotoxic stimuli[3]. The signaling pathways of apoptosis and necroptosis will be discussed in the following sections. Although stress stimuli may also induce autophagy which is a catabolic process to remove protein aggregates and damaged organelles for recycling[4], this process is not described in the manuscript here since it may lead to either cell survival or apoptotic death.

Apoptosis

Apoptosis is a type of programmed cell death that is characterized by morphological and ultrastructural changes, including cell shrinkage, membrane blebbing, mitochondrial swelling, and chromatin condensation. Apoptosis may either be initiated extrinsically *via* death receptors such as tumor necrosis factor (TNF) receptor and Fas, or intrinsically *via* mitochondria-dependent pathways[5] (Figure 1). Moreover, anoikis, which is a form of detachment-induced apoptosis, has been demonstrated to occur in epithelial cells, as they normally require anchorage to basement membranes to establish a monolayer[6].

In the extrinsic apoptotic pathway, the recruitment of cytoplasmic molecules to receptors is initiated following the binding of TNFα or FasL. Docking molecules, including TNF receptor-associated death domain (TRADD), Fas-associated death domain (FADD), procaspase-8/FLICE/MACH, and receptor-interacting protein kinase (RIPK)-1, are recruited to receptor-associated lipid rafts to form a complex that facilitates the cleavage and activation of caspase-8[7,8]. The intrinsic apoptotic pathway occurs following endogenous stress and is associated with a drop in mitochondrial membrane potential. This pathway is regulated by the formation of a mitochondrial permeability transition pore (MPTP), which is composed of Bcl-2 family members and voltage-dependent anion channels on the outer mitochondrial membrane[5,9]. The ratio of Bcl-2 family proteins (*i.e.,* anti-apoptotic Bcl-2 and Bcl-X_L and pro-apoptotic Bax, Bad, Bak, Bid, Bim, and PUMA) is a key factor in determining the conformation of MPTP. Among the Bcl-2 members, Bid can be cleaved by caspase-8 and migrate to the mitochondria in its truncated form tBid to associate with Bax to increase membrane permeability. The drop of mitochondrial membrane potential leads to osmotic swelling of the matrix by water influx and release of cytochrome c from mitochondrial intramembranous space into the cytoplasm, followed by its complex formation with procaspase-9 and APAF-1. The activation of caspase-9 and/or -8 leads to caspase-3 cleavage, endonuclease activation, and ultimately nuclear DNA fragmentation, which is the hallmark of apoptosis[5,9] (Figure 1).

Regulatory proteins, such as FLICE-Like Inhibitory Proteins (FLIPs), inhibit the extrinsic apoptotic pathway by binding to FADD and causing dissociation of the FADD-caspase 8 complex. Additionally, families of inhibitor of apoptosis protein (IAP), including XIAP, cIAP, and survivin, bind to caspase-3 and -9 and thereby inhibit caspase activity. Moreover, XIAP-associated factor 1 (XAF1) negatively regulates the anti-apoptotic function of XIAP[5,9,10].

Necrosis

Necrosis is traditionally known as an uncontrolled form of cell death, characterized by morphological features of mitochondrial swelling, cytoplasmic vacuolation, cytosol density loss, and plasma membrane rupture. The resultant release of subcellular organelles and molecules is considered a potent trigger for tissue inflammation[11].

Necroptosis

A novel form of programmed necrosis, termed necroptosis, has been recently identified. In this pro-

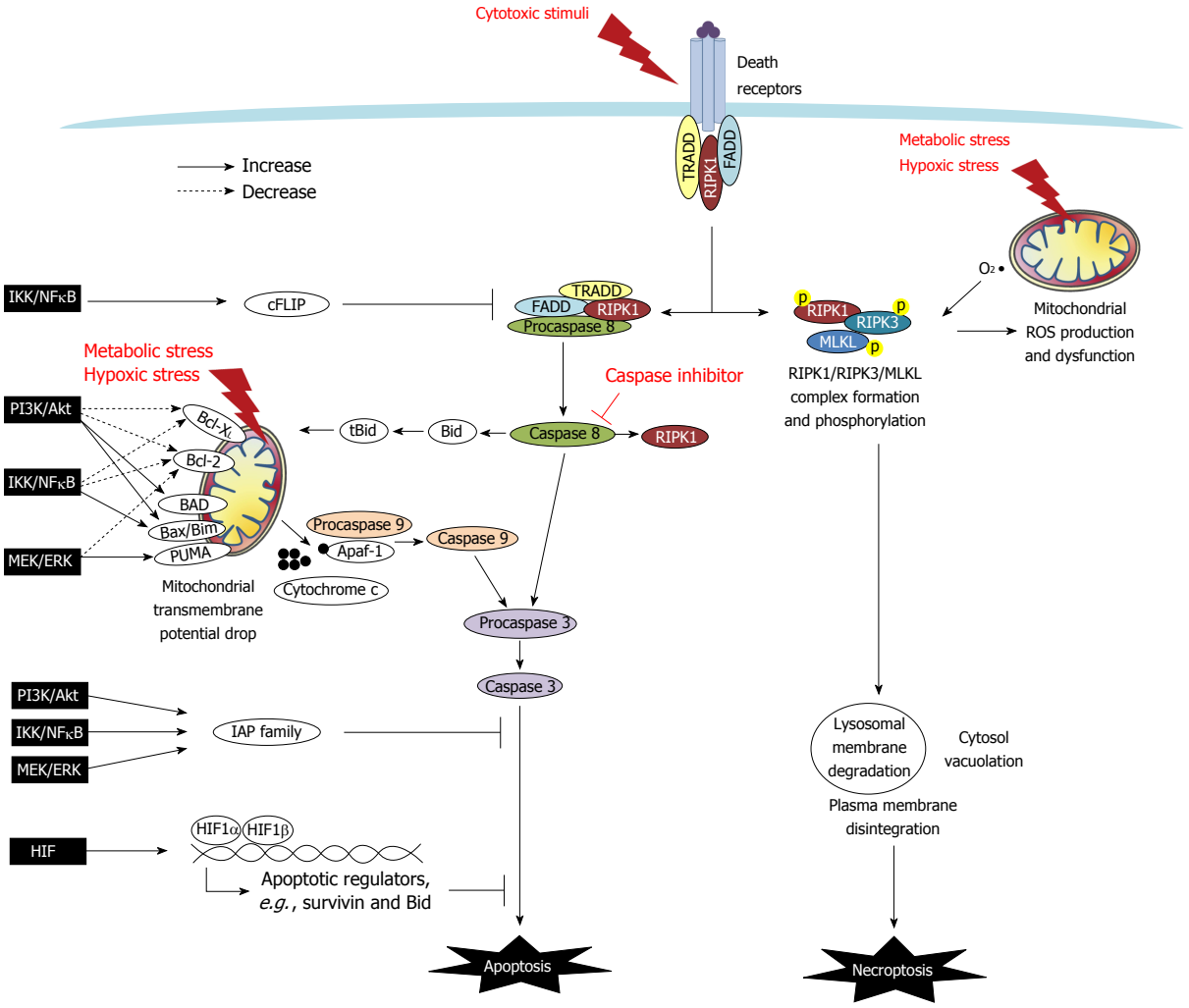

Figure 1 Death resistance signaling in cancer cells. Programmed cell death (*i.e.*, apoptosis and necroptosis) are either triggered extrinsically by cytotoxic stimuli through death receptors, or initiated intrinsically *via* mitochondria dysfunction caused by metabolic and hypoxic stress. In the extrinsic apoptotic pathway, tumor necrosis factor (TNF) or Fas binding to the receptors trigger the recruitment of adaptor molecules to form a death-inducing signaling complex which contains TNF receptor-associated death domain (TRADD), Fas-associated death domain (FADD), procaspase 8/FLICE, and receptor-interacting protein kinase 1 (RIPK1) to facilitate the activation of caspase 8. Caspase 8 then cleaves and activates caspase 3 (the final caspase in the apoptotic pathways), and it also truncates Bid and RIPK1. The intrinsic apoptotic pathway is associated with mitochondrial dysfunction. The ratio of Bcl-2 superfamily proteins, including anti-apoptotic Bcl-XL and Bcl-2, and pro-apoptotic Bad, Bid, Bax, Bim and PUMA, determines the formation of the mitochondrial permeability transition pore. The truncated form tBid cleaved by caspase-8 can migrate to the mitochondria to associate with Bax to increase membrane permeability. The drop of mitochondrial transmembrane potential leads to osmotic swelling and release of cytochrome c to complex with Apaf-1 and procaspase 9 which undergo cleavage into the active form of caspase 9. Caspase 9 and/or caspase-8 activates caspase-3, and ultimately leads to nuclear DNA fragmentation. Moreover, FLICE-like proteins (FLIP) and inhibitors to apoptosis proteins (IAPs), including cIAP, survivin and XIAP, provide a brake on the apoptotic cascade. In cancers, signaling pathways such as PI3K/Akt, MEK/ERK, IKK/IκB/NFκB and HIF regulate apoptosis by modulating Bcl-2 members and altering expression of FLIP and IAPs. In the extrinsic necroptotic pathway, stimulus of TNFα in the presence of a caspase inhibitor frees RIPK1 to form a complex with RIPK3 for auto- and trans-phosphorylation, which then recruits and phosphorylates MLKL. The RIPK1/RIPK3/MLKL complex causes mitochondrial dysfunction and executes subcellular features of necroptosis, such as lysosomal membrane degradation, cytosol vacuolation, plasma membrane disintegration, and ultimately cellular explosion. In the intrinsic necroptotic pathway, metabolic and hypoxic stress induces the mitochondrial production of reactive oxygen species (ROS) such as superoxide, which subsequently leads to RIPK1/3 activation and the final steps of necroptosis. However, signaling pathways to regulate necroptosis has not yet been reported.

cess, signaling pathways involving RIPK1/RIPK3-mediated phosphorylation activate the mixed lineage kinase domain-like protein (MLKL) to execute the final step of cell destruction[11] (Figure 1). The best defined necroptosis pathway was elucidated following the stimulation of cells with TNFα in the presence of ZVAD (a pan-caspase inhibitor)[11,12]. This observation has led to the development of a preliminary hypothesis that necroptosis may be a default mechanism for cells that are unable to die *via* apoptosis[12,13]. However, with increasing evidence of instances of necroptotic death occurring following various stimuli (*e.g.,* oxygen and glucose deprivation, extensive DNA damage, hyperactivation of Poly(ADP-ribose)polymerase -1 (PARP), and free radical exposure), it is now clear that RIPK1/3-dependent necrosis is an independent mode of cell death that shares common pathways with apoptosis[14,15].

Early studies of necroptotic pathways by activating TNF receptor in the presence of caspase inhibition

demonstrated that the adapter molecules FADD and TRADD recruited RIPK1, which subsequently undergo a series of ubiquitination and deubiquitination events before RIPK1 forming a complex with RIPK3 and MLKL in the cytosol for auto- or trans-phosphorylation[16]. The RIPK1/RIPK3/MLKL kinase complex has been proposed to mediate necrotic death *via* the induction of mitochondrial dysfunction[16]. Additionally, mitochondria-derived free radical production and lysosomal membrane disintegration have been reported to be facets of necroptotic machinery[17]. Free radical scavengers that suppress mitochondrial reactive oxygen species (ROS) were shown to inhibit the execution of necroptotic death induced by TNF and hypoxic stress, but had no effect to necroptosis induced by PARP[18,19]. Other reports revealed that hypoxia-induced mitochondrial ROS was upstream of RIPK1/RIPK3 activation in the necroptotic signaling pathway[19]. The order of intracellular events leading up to plasma membrane explosion and intracellular content spilling may vary depending on trigger type[17-19]. Overall, it is currently recognized that two modes of cell death are driven by RIPK1 through its kinase function, including apoptosis *via* its formation of a complex with caspase-8/FADD/TRADD and necroptosis *via* its formation of a complex with RIPK3/MLKL (Figure 1).

APOPTOSIS IN NORMAL COLON AND COLORECTAL CANCERS

Physiological cell turnover in intestine

The intestinal epithelial monolayer is maintained in a state of dynamic equilibrium that is governed by the balance between crypt proliferation and surface/villus shedding and cell death. Newly proliferated cells that are derived from stem cells in the crypts migrate upward and differentiate into various cell types (*e.g.,* absorptive and secretive epithelial cells, goblet cells, and endocrine cells); the cells then undergo detachment and apoptosis at an "extrusion zone" on the luminal surface with a turnover rate of 5-7 d[20,21]. Epithelial integrity and intestinal homeostasis are tightly controlled by the balancing of two physiological processes, namely cell proliferation and death.

Inverse correlation between epithelial apoptosis and colon tumor susceptibility

Progressive inhibition of cell apoptosis has been associated with the transformation of normal colorectal epithelium into carcinoma[22]. Direct evidence of an inverse correlation between epithelial cell death and tumor susceptibility has been provided in recent studies. Mice that were deficient in pro-apoptotic molecules (*e.g.,* Bak and Fas) displayed a higher incidence and higher numbers of aberrant crypt foci and colorectal tumors following induction with the carcinogen azoxymethane (AOM) or AOM/dextran sulfate sodium (DSS)[23,24]. Although a lack of Bak or Fas did not affect physiological apoptosis in colon cells, a decreased level of epithelial cell death was observed following exposure to pro-apoptotic triggers (*e.g.,* gamma-radiation and genotoxic carcinogens)[23,24]. Moreover, PUMA-knockout mice exhibited reduced apoptosis in colonic crypts and increased colonic tumor susceptibility following an AOM/DSS challenge. A deficiency of PUMA enhanced the formation of spontaneous adenomas in the distal small intestines and colons of APC(Min/+) mice[25]. These studies indicate that cells that are unable to undergo apoptosis partly contribute to cancer progression.

ANTI-DEATH CYTOPROTECTIVE STRATEGIES IN NORMAL INTESTINE

Surface epithelial layers are constantly bombarded by orally acquired harmful substances and luminal bacteria, and are also exposed to potentially hypoxic conditions due to their location at the end of a capillary circuit that interfaces with an anaerobic lumen. When exposed to intrinsic stress or external stimuli, a normal epithelium exhibits excessive cell death (*i.e.,* apoptosis, necrosis and necroptosis) and display barrier defects. However, cytoprotective strategies against cell death also exist to maintain gut homeostasis.

The cellular survival strategies include uptake of glucose and glutamine, free radical scavenging, transcriptional adaptation, and paracrine effects induced by cyclooxygenase (COX)-2/prostaglandin E2 (PGE2). In the following sections of this work, several facets of anti-death cytoprotective strategies that share common pathways to tumor resistance will be discussed. Understanding the similarity between epithelial cytoprotection and cancer resistance would help to identify distinct mechanisms undertaken by tumor cells for the search of advanced therapeutic targets.

One of the unique characteristic of the intestinal tract is that it possesses dual routes of nutrient supply, including hematologic and dietary sources. In small intestinal epithelium, apical glucose uptake is mediated by sodium-dependent glucose transporter 1 (SGLT1), while glucose transporter 2 (GLUT2) facilitates diffusive transport of intracellular glucose across the basolateral membrane and into the bloodstream[26]. Large intestinal epithelium normally expressed GLUT5 and GLUT6, but not the other glucose transporters[27]. We and others have previously shown that enhanced glucose uptake *via* SGLT1 can protect intestinal epithelial cells against various pro-apoptotic triggers, such as mesenteric ischemia/reperfusion, microbial challenges, and endotoxemia[28-31]. Energy production has been generally assumed as the main cytoprotective mechanism of glucose uptake; however, alternative pathways of SGLT1-mediated activation of phosphatidylinositide 3-kinase (PI3K)/Akt and nuclear factor kappa B ($NF\kappa B$) pathways also partially contributes to cytoprotection[28,31]. Other than glucose,

glutamine (a non-essential amino acid) is important for cell survival during conditions of stress. Glutamine can prevent epithelial cell apoptosis caused by hypoxia/reoxygenation, oxidants, endotoxins, heat stress, and TNFα[32-36]. The inhibition of gut epithelial cell apoptosis by glutamine is mediated through upregulation of autophagy and increased transcription of heat shock proteins[34,35,37].

Redox enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione reductase, and glutathione-S-transferase, suppress intracellular accumulation of free radicals. An intracellular redox system converts highly reactive free radicals, such as superoxide, hydrogen peroxide, and hydroxyl radical, into lower energy molecules. Intravenous injections of CAT and SOD have been shown to decrease intestinal inflammation and epithelial barrier dysfunction in animal models of mesenteric ischemia/reperfusion injury[38-40].

Hypoxia-inducible factor (HIF) is a transcription factor that is activated in epithelial cells under the low oxygen conditions of ischemic or inflamed gut[41,42]. The HIF family includes three proteins: HIF-1, HIF-2, and HIF-3. Activation of HIF is dependent on the stabilization of the oxygen-sensitive α subunit, which is subsequently translocated to the nucleus to form a functional complex with the β subunit and various other coactivators[43]. HIF-1α/2α forms a dimeric complex with HIF-1β, and triggers transcription by binding to the hypoxia response element of various gene promoter regions[44,45]. Under normoxic conditions, the hydroxyl hydroxylase (PHD)-mediated hydroxylation of proline residues on HIF-1α/2α leads to its ubiquitination and degradation. Low oxygen levels have been shown to result in a downregulation of PHD activity and to stabilize HIF-1α/2α levels[43]. HIF-1 activation has been implicated in maintaining epithelial barrier protection in models of intestinal ischemia/reperfusion, experimental colitis with inflammatory hypoxia, and in mouse ileal loops after exposure to bacterial toxins[41,42,46-48].

Cyclooxygenase (COX)-2, a catalyzing enzyme for PGE2 production, is involved in increased vascular permeability and blood flow during inflammation and wound healing. COX-2 also protects against colonic epithelial damage in animal models of chemical-induced colitis[49,50]. Oral supplementation of PGE2 increased proliferation and reduced apoptosis of intestinal epithelium in mice with colitis[49]. Moreover, PGE2 increased c-IAP2 expression in normal rat epithelial cells[51].

The detailed anti-death signaling pathways have primarily been delineated from observations made in adenocarcinoma cell line studies, and they will be discussed in the context of cancer cell death resistance in the next section.

INTRACELLULAR MECHANISMS OF DEATH RESISTANCE IN CANCERS

Death and anti-death signaling in cancer cells has been extensively studied in the context of cell survival against exogenously applied cytotoxic drugs. Substantial efforts have also been made to uncover the pathways that lead to cancer cell death and survival under conditions of endogenous metabolic stress, with the goal of learning how to therapeutically manipulate tumor-specific machinery to produce anti-cancer effects. We will highlight the mechanisms of death resistance in cancer cells against exogenous or endogenous stress. These intracellular pathways include direct alteration of death machinery and modulation of upstream events such as tumor suppressor gene inactivation and pro-survival signaling. For additional discussion of drug-related resistance mechanisms, such as efflux pumps and enzymatic degradation, please see other review articles[52,53].

Direct alteration of apoptotic and necroptotic regulators in cancer

Defects in apoptotic signaling and increased use of anti-apoptotic pathways have been reported in colon cancer cells. Key regulatory proteins of apoptotic machinery, such as families of Bcl-2 and IAP, undergo changes in expression during the transition of an adenoma into a carcinoma and have therefore been utilized as prognostic biomarkers[54,55]. An overexpression of the anti-apoptotic Bcl-2 family member Bcl-X_L is a known predictor of poor prognoses in patients with colonic adenocarcinomas[56]. Increased expression levels of anti-apoptotic regulators such as c-FLIP, XIAP, cIAP2, and survivin have also been correlated with disease progression and poor survival in colon cancer patients[57-61]. A recent report indicated that expression levels of the necroptotic adaptors RIPK1 and RIPK3 are significantly decreased in human colon cancer tissues compared to adjacent normal mucosa[62]. Overall, an increase of anti-apoptotic molecules and a decrease in pro-necroptotic kinases are both likely to contribute to death resistance in cancer cells (Table 1). The resistance mechanisms that occur upstream of the alteration of apoptotic regulators are further discussed below.

Inactivation of tumor suppressor genes to prevent apoptosis in cancer

Mutations in oncogenes (RAS and β-catenin) and tumor suppressor genes (p53 and APC) have been identified to arise throughout the course of tumorigenesis[63]. The intriguing field of oncogene mutation and cell hyperproliferation has been reviewed comprehensively[64] and will not be discussed here. Instead, cancer cell death resistance that is imparted by mutation of the p53 tumor suppressor gene and the resultant functional consequences of this resistance will be the focus of this section.

Mutations in the p53 gene occur in half of all colorectal cancer cases and have been correlated with adenoma-to-carcinoma transitions and aggressive subsets of colorectal cancer[65,66]. Tumor cells harboring

Table 1 Alteration of apoptotic and necroptotic regulators in human colon cancers

Classification	Molecule	Expression in cancer tissues	Ref.
Bcl-2 family	Bcl-X_L	Increased	[56]
IAP family	cFLIP	Increased	[57]
	cIAP2	Increased	[59,60]
	survivin	Increased	[55,61]
	XIAP	Increased	[58]
RIP kinase family	RIPK1/RIPK3	Decreased	[62]

IAP: Inhibitor of apoptosis protein; FLIP: FLICE-like inhibitory protein; XIAP: X-linked IAP; RIP: Receptor-interacting protein.

p53 mutations have long been known to be defective in the induction of apoptosis[67]. In addition to its pro-apoptotic role, it has become evident that p53 acts as a multifunctional transcription factor and is involved in physiological cellular responses to stressful stimuli (*e.g.,* DNA damage and hypoxia), surveillance mechanisms that cause cell cycle arrest following cellular damage or oncogenic aberration, and regulation of metabolic pathways for a switch from glycolysis to oxidative phosphorylation[68,69]. The molecular mechanisms that are employed by p53 to induce cell death in the context of suppressing cancer progression include the transcriptional regulation of pro-apoptotic PUMA expression, the generation of oxidative free radicals within mitochondrial components, the reduction of COX-2/PGE2 synthesis, and the induction of death receptor 5[70-73].

Signaling pathways that modulate apoptotic regulators in cancer

A number of signaling molecules and transcription factors are involved in the dysregulation of death machinery in cancer cells (Figure 1). These include the PI3K/Akt, mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), IκB kinase (IKK)/inhibitor of NFκB (IκB)/NFκB, and HIF signaling pathways (Table 2).

Studies on colon cancer cell lines have shown that an upregulation of PI3K/Akt protein kinases has been associated with increased expression of anti-apoptotic Bcl-2 proteins (*e.g.,* Bcl-2, Bcl-X_L, and survivin)[74,75], phosphorylation and inactivation of pro-apoptotic Bad and Bax[76-78], and activation of XIAP[79]. In colon cancer and epithelial cells, the MEK/ERK signaling pathway mediates the phosphorylation and stabilization of Bcl-2[80], the inactivation of Bax and the degradation of Bim[78,81], the suppression of PUMA induction[73], the downregulation of XAF1 and the upregulation of XIAP expression[82].

The activation of the IKK/IκB/NFκB pathway, which serves as a putative proinflammatory signal, has been linked with anti-apoptotic effects in normal colonocytes and colon cancers alike. The activation of the IκB kinase complex (IKKα/β/γ) leads to the phosphorylation of NFκB-bound IκB and causes

Table 2 Signaling pathways for modulation of apoptotic regulators in cancer

Pathway	Observation in experimental models	Ref.
PI3K/Akt	Increase of Bcl-2, Bcl-X_L and survivin expression in colon cancer cell lines (SW480, SW620, HCT116, and HT29)	[74,75]
	Inactivation of BAD by phosphorylation in colon cancer cell lines (HT29 and H508)	[76,77]
	Decreased expression of Bim and inactivation of Bax in colon cancer cell lines (HCT116 and DLD1), and increase of tumor growth in xenograft models	[78]
MEK/ERK	Phosphorylation and stabilization of Bcl-2 in colon cancer cell lines (HCT116 and HT29) for increase of anoikis, and promotion of metastasis in xenograft models	[80]
	Decreased expression of Bim and inactivation of Bax in colon cancer cell lines (HCT116 and DLD1), and increase of tumor growth in xenograft models	[78]
	Suppression of PUMA expression and activity in colon cancer cell lines (Lovo and SW1116)	[73]
	Dowregulation of XAF1 and upregulation of XIAP in colon cancer cell lines (HCT116, Lovo, DLD1, and SW1116)	[82]
IKK/IκB/NFκB	Induction of cIAP-2 expression in colon cancer cell lines (Caco-2, HCT116, KM20, and KM12C)	[91]
	Increase of Bcl-2, Bcl-X_L, and cFLIP in colon cancer cell lines (COLO205 and HCT116)	[92]
HIF	Binding to hypoxia-responsive element of the Bid promoter in colon cancer cells (SW480) for Bid downregulation	[94]
	Binding to hypoxia-responsive element of the survivin promoter in breast cancer cells (MCF-7) for survivin upregulation	[93]

PI3K: Phosphatidylinositide 3-kinase; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated kinase; NFκB: Nuclear factor kappa B; IκB: Inhibitor of NFκB; IKK: IκB kinase; HIF: Hypoxia-inducible factor.

IκB to undergo ubiquitin-dependent degradation, enabling the liberated NFκB to translocate to the cell nucleus and act as a transcription factor[83]. Unlike other subunits in the complex, IKKα can shuttle between the nucleus and cytoplasm to facilitate NFκB-regulated gene expression[84]. An elegant study indicated that either epithelial-specific ablation of IKKγ (also called NEMO) or a deficiency of both IKKα and IKKβ can lead to increased levels of apoptosis in mouse colonocytes[85]. Using carcinogen-induced colon cancer models, epithelial-specific IKKβ-KO mice were shown to exhibit increased colonic cell apoptosis associated with a reduction of tumor growth compared to wild type mice[86]. Numerous studies of colonic, gastric and esophageal cancer cell lines have demonstrated that blocking NFκB-induced apoptosis under baseline conditions sensitizes cells to treatment with 5-fluorouricil (5-FU)[87-89]. The underlying mechanisms of this phenomenon included IKKα-mediated phosphorylation of CREB binding protein (a transcriptional coactivator), which induced a switch in binding preference from p53 to NFκB and led to a concurrent upregulation of NFκB-dependent anti-apoptotic genes, and the downregulation of p53-

mediated pro-apoptotic genes[90]. Moreover, in colon cancer cells, the transcriptional targets of NFκB include the anti-apoptotic regulators Bcl-2, Bcl-X_L, cFLIP and IAP[91,92].

HIF is upregulated in the hypoxic core of rapidly growing solid tumors and is therefore considered a biomarker of poor prognosis in colon cancer patients[45]. HIF-1 targets promoter sites of apoptotic regulators such as survivin and Bid, and directly modulates cell death pathways[93,94]. Other HIF-1-targeted genes included trefoil factor[47], COX-2[95], glucose metabolic enzymes [*e.g.,* hexokinase (HK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK), pyruvate dehydrogenase kinase (PDK)], and glucose transporters (*e.g.,* GLUT-1 and -3)[45,96,97]. HIF-1α also directly activates promoter regions of various growth factors and receptors, including vascular endothelial growth factor (VEGF), c-Met [a receptor for hepatocyte growth factor (HGF)], HGF activator[98-100]. Moreover, several reports have shown that HIF-2α increases transcriptional and translational expression of amphiregulin [a member of the epidermal growth factor (EGF) family] and EGF receptor (EGFR), and favors autocrine growth signaling in various types of cancer[101-103].

TUMOR MICROENVIRONMENT CAUSES DEATH RESISTANCE IN COLON CANCER

Autocrine, paracrine and exogenous factors in tumor microenvironments also instigate resistance against apoptotic and necroptotic cell death in colon cancers *via* changes in receptor signaling and transporter uptake (Figure 2). The roles of COX-2/PGE2, growth factors, glucose, and bacterial LPS are highlighted below.

COX-2/PGE2-mediated apoptotic resistance in cancer

Increased expression of COX-2 was observed in colon adenocarcinoma, and is considered one of the earliest events in tumor development[104,105]. Clinical studies have provided strong evidence that long term use of non-steroidal anti-inflammatory drugs reduce the incidence of colon cancers[106]. Various mechanisms have been proposed for the COX-2-mediated tumor-promoting activity, including increased blood flow, induction of growth factors, increase of cell proliferation, or modulation of apoptotic regulators[51,107,108]. COX-2/PGE2 induces synthesis of amphiregulin and activation of EGFR signaling in colon cancer cell lines[107,108]. Moreover, stimulation with PGE2 was found to suppress Fas-induced apoptosis in colon cancer cells *via* upregulation of IAP expression[51] (Figure 2).

Growth factor-dependent apoptotic and necroptotic resistance in cancer

A number of growth factors have been associated with death resistance in cancer, including epidermal, hepatocyte, and insulin-like growth factors (Figure 2). EGFR activation has long been known to induce epithelial cell proliferation, restitution, and tumorigenesis. In colorectal and gastric cancer cells, EGFR activation also exerts anti-apoptotic effects that are mediated by PI3K/Akt, ERK and IκBα/NFκB signaling, and protects cancer cells against anoikis and enhances epithelial-mesenchymal transition[109-112]. Moreover, both EGFR and its downstream Akt and ERK pathways are known to be involved in preventing apoptosis in stem-like cell populations in serum-deprived colorectal cancer cells[113]. A positive feedback loop between EGFR and HIF signaling pathways may also contribute to the increase of death resistance in cancer, as revealed by evidence that EGFR activation leads to increased expression and nuclear translocation of HIF-1α/1β in normoxic conditions[93,95,114]; furthermore, HIF-2α increases the transcript and protein expression of EGFR and amphiregulin[101-103]. Recent evidence has revealed a co-expression of EGFR and SGLT1 in human colorectal and oral squamous carcinoma[115,116]. Interactions between EGFR and SGLT1 have shown a positive correlation with cancer cell proliferation and survival through a mechanism that involves the stabilization of membranous SGLT1 proteins by EGFR in a tyrosine kinase-independent manner[117,118].

Elevated expression of the HGF receptor Met has been shown in human colorectal cancers compared to normal mucosa[119]. Recently, both the autocrine production of HGF and a positive feedback loop that was mediated by a constant activation of the HGF gene promoter due to microsatellite instability that was characterized by truncations of the promoter region were found in human colon carcinoma samples and cell lines[120]. Activation of HGF/Met signaling was shown to cause a downregulation in the expression of RIPK1 proteins, which correlated with a reduction in the number of necroptotic regions found in colon tumors[120]. Moreover, evidence of HIF-1α directly activating the promoter regions of Met and HGF activator (a serine protease that converts HGF to its active form) was found in pancreatic cancer and glioma cells[99,100]. Interestingly, increased activation of Met was found to be induced in human carcinoma Caco-2 cells following treatment with cetuximab (an EGFR inhibitor), which served as a chemoresistance mechanism[121].

Colon cancers express high levels of both insulin-like growth factor 1 (IGF1) and its receptor compared to normal mucosa. The overexpression of IGF1 receptors in human colon cancer cells was found to confer resistance to serum-deprivation induced apoptosis, which was associated with increased activation of Akt and an upregulation of Bcl-X_L[122]. Another report demonstrated that inhibition of the IGF1 receptor sensitized human colon adenocarcinoma cells to cetuximab and restored cell death in drug-resistant cell clones[123].

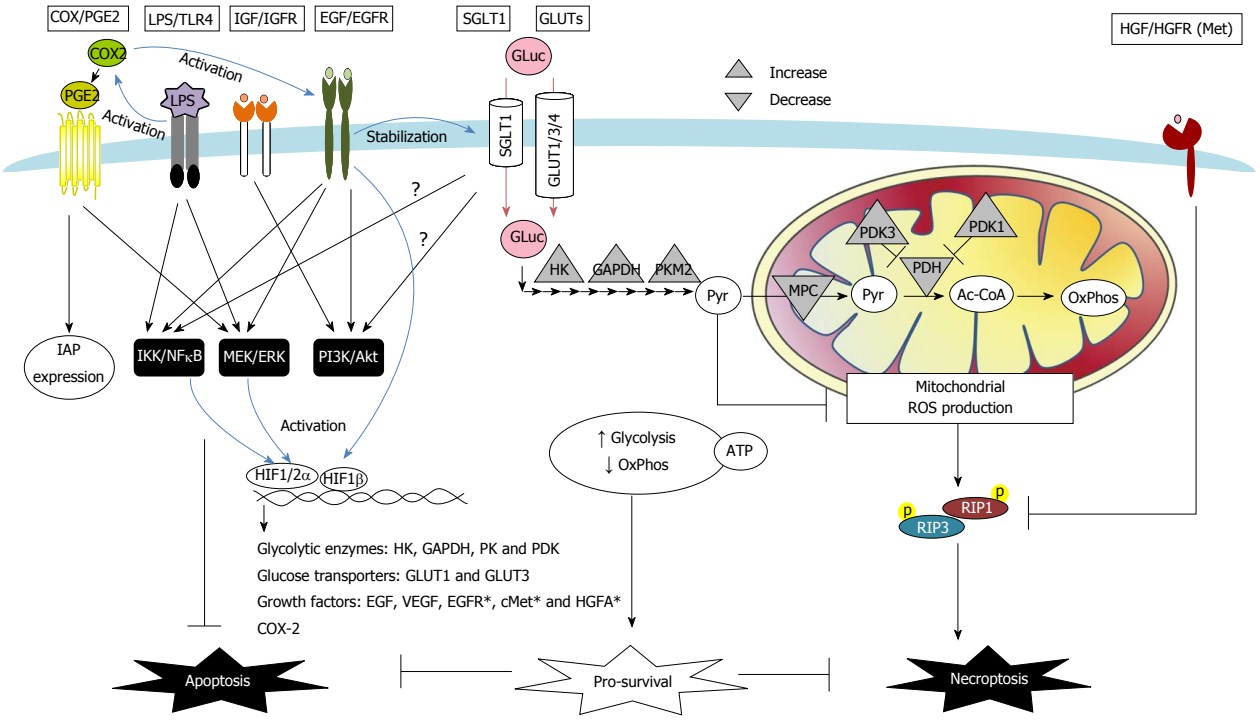

Figure 2 Proposed schema of death desistance mechanisms *via* modulation of receptor signaling and transporter uptake in colon cancer cells. A number of autocrine, paracrine, or exogenous factors instigate death resistance in colon carcinoma. These pathways included cyclooxygenase (COX)-2/prostaglandin E2 (PGE2), bacterial lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4), growth factors [*i.e.*, insulin-like growth factor (IGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF)], as well as glucose transport and metabolism. COX/PGE2 upregulates the IAP expression and activates EGF/EGFR signaling to inhibit apoptosis in colon cancer cells. TLR4 antagonizes cell apoptosis caused by its co-receptor CD14, induces anti-apoptotic MEK/ERK and IKK/IκB/NFκB signaling, and activates COX-2 pathways in colon carcinoma. Growth factors such as IGF and EGF induce anti-apoptotic PI3K/Akt, MEK/ERK, and IKK/IκB/NFκB pathways in colon cancers. Moreover, activation of HGF and its receptor Met renders colon cancer cells resistant to necroptosis *via* downregulation of RIPK1 protein expression. Alteration of transport and metabolism of glucose (Gluc) is another survival strategy of cancer cells. Abnormally expressed sodium-dependent glucose transporter 1 (SGLT1) and GLUT1/3/4 enhance glucose uptake in colon carcinoma. Activation of SGLT1 induces PI3K/Akt and IKK/IκB/NFκB pathways in normal intestinal epithelial cells; however, their roles in anti-death mechanisms of colon cancers remain unclear (?). Increased glycolysis and decreased mitochondria-dependent oxidative phosphorylation (OxPhos) are commonly seen in cancer cells. The metabolic shift results from upregulated expression of glycolytic enzymes for increased (Pyr) production [*e.g.*, hexokinase (HK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK)], and also from downregulated expression of mitochondrial pyruvate carrier (MPC) and pyruvate dehydrogenase (PDH) that limits pyruvate conversion to Acetyl Co-A (Ac-CoA). The metabolic shift and predominantly glycolytic ATP generation are adaptive responses to hypoxic stress and promotes cancer cell survival. The final glycolytic product pyruvate, which is also a free radical scavenger, prevents hypoxia-induced necroptotic death in colon cancer cells *via* suppression of mitochondrial ROS. Hypoxia acts a stressor but also a death regulator by HIF-dependent transcription of a number of genes, including glucose metabolic enzymes [*e.g.*, HK, GAPDH, PK, and pyruvate dehydrogenase kinase (PDK)], glucose transporters (*e.g.*, GLUT-1 and GLUT-3), and growth factors [*e.g.*, EGF and vascular endothelial growth factors (VEGF)]. Other HIF-targeted genes, *e.g.*, EGFR, cMet, and HGF activator (HGFA), were reported on non-intestinal cancer cells (*), and may also contribute to the death resistance mechanisms. Lastly, EGF activates HIF signaling in normoxic conditions, leading to a positive feedback loop of adaptation fueling anti-death and pro-proliferative cancer growth.

Glucose-dependent apoptotic and necroptotic resistance in cancer

Increased glucose dependency and altered glucose metabolism are associated with cancer cell transformation (Table 3). In normal cells, one glucose molecule is catalyzed into two ATPs and two pyruvate molecules in an anaerobic fashion by a cascade of glycolytic enzymes, including HK, GAPDH, and PK[124]. The final glycolytic product, pyruvate, is transported across the inner mitochondrial membrane (IMM) by mitochondrial pyruvate carrier (MPC) and is then converted to acetyl-CoA by pyruvate dehydrogenase (PDH) before entering into the tricarboxylic acid (TCA) cycle that occurs in the mitochondrial matrix[125,126]. A reduced substrate is generated by the TCA cycle and is then fed into the electron transport chain of the IMM, after which oxidative phosphorylation leads to the production of 36 ATPs. In contrast to normal cells, tumors exhibit high levels of glycolysis despite the presence of sufficient oxygen, which is a phenomenon known as the Warburg effect[127].

The abnormal expression of the GLUT isoforms 1, 3, and 4 and the SGLT1 protein have been widely documented in studies of human colon cancer samples[27,115,116,128-130]. A large body of evidence indicates that the upregulation of GLUT1 and several glycolytic enzymes is dependent on the transcriptional activity of HIF-1 in both human colon cancer tissues and drug-resistant cancer cell lines[96,131-133]. Recent studies have indicated that the stabilization of membrane SGLT1 expression in colon cancer cells is dependent on EGFR activation[117,134], suggesting that this pro-proliferative and anti-apoptotic growth factor is also involved in the mechanism that underlies enhanced glucose uptake (Figure 2).

Changes in glucose uptake and metabolism have

Table 3 Glucose-dependent mechanisms in death resistance of colon cancer

Classification	Molecule	Expression and mechanism	Ref.
Glucose uptake			
Transporters	GLUT1	Abnormal expression of GLUT1 in colon cancer	[128,129]
		Hypoxia-induced expression of GLUT1 by HIF-1 binding to the GLUT1 promoter	[96,131,132]
		GLUT1-mediated glucose uptake promoted drug resistance in colon cancer cells	[136]
	GLUT3,4	Abnormal expression of GLUT3,4 in colon cancer	[19,27,130]
	SGLT1	Abnormal expression of SGLT1 in colon cancer	[27,115]
		Stabilization of membrane SGLT1 expression is dependent on EGFR in a kinase-independent mechanism	[117,134]
Glucose metabolism			
Enzymes	PK	Upregulation of PKM2 isoform in chemoresistant cancer cells	[132]
	PDK-1	PDK-1 as a novel Wnt target gene improved colon cancer cell survival via enhancement of glycolysis	[142]
	PDK-3	HIF1-mediated upregulation of PDK-3 inhibited mitochondrial phosphorylation and promoted drug resistance	[143]
	HK, GAPDH	HIF1-dependent transcriptional upregulation	[45,96]
	PDH	Decreased expression in colon cancer cells	[141]
Carriers	MPC	Reduction of MPC activity promoted glycolysis and maintenance of stemness properties	[144]
Products	ATP	Elevation of intracellular ATP promoted cancer cell survival and induced drug resistance	[145,146]
	Pyruvate	Pyruvate prevented hypoxia-induced necroptosis through suppression of mitochondrial free radicals in an ATP-independent mechanism	[19]

GLUT: Glucose transporter; SGLT1: Sodium-dependent glucose transporter 1; EGFR: Epidermal growth factor receptor; PK: Pyruvate kinase; PDK: Pyruvate dehydrogenase kinase; HK: Hexokinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PK: Pyruvate kinase; PDH: Pyruvate dehydrogenase; MPC: Mitochondrial pyruvate carrier.

been suggested to provide a survival advantage to tumor cells and also to contribute to anti-cancer drug resistance. A previous report has shown that high glucose levels can modulate the cytotoxicity and anti-proliferative effects of 5-FU in colon cancer cell lines[135]. The *in vitro* inhibition of GLUT1-mediated glucose uptake by phloretin was found to sensitize colon cancer cells to overcome apoptotic resistance to daunorubicin under conditions of hypoxia[136]. The modulation of glucose metabolic pathways by chemicals (*e.g.,* 3-bromopyruvate, iodoacetate, dichloroacetate, or 2-deoxyglucose) was found to attenuate 5-FU resistance in colonic and gastric cancer cells[137-139]. Recent studies have indicated that high levels of intracellular glucose lead to an increased side population (SP) of stem-like cells within the overall cancer cell population; these cells exhibit high glycolytic activity and drug export ability, and are most resistant to cell death[140]. The underlying mechanism of SP cell expansion involves glucose-mediated suppression of AMP-activated protein kinase and an activation of Akt signaling[140].

PK is the final rate-limiting enzyme in the glycolytic pathway, and it catalyzes the conversion of phosphoenolpyruvate and ADP into pyruvate and ATP. PDH, which converts pyruvate into acetyl-CoA, can be phosphorylated and inactivated by PDK in physiological conditions. The PK isoform M2 (PKM2) is significantly upregulated in 5-FU-resistant colon cancer cell lines[132]. Moreover, decreased expression of PDH was reported in colorectal cancer cells[141]. A recent study has identified that PDK-1 is a novel Wnt target gene that can promote glycolysis and colon cancer cell survival[142]. Hypoxia-induced PDK-3 expression *via* the activation of HIF-1 has been shown to lead to an inhibition of mitochondrial phosphorylation and the promotion of drug resistance[143]. Additional research has indicated that reduced MPC activity promotes glycolysis and the maintenance of stemness in colon cancer cells[144].

ATP has generally been assumed to be the effector of glucose metabolism that promotes cancer growth, survival, and chemoresistance. A number of studies have shown that depleting ATP *via* glycolytic inhibition increases apoptosis in multidrug- resistant cancer cells under both normoxic and hypoxic conditions[145,146]. Moreover, direct delivery of liposome-encapsulated ATPs was found to be sufficient for promoting survival and inducing resistance to oxaliplatin in formerly drug-sensitive colon cancer cells[146]. Whether ATP-dependent chemoresistance is a result of cell hyperproliferation or the induction of anti-death mechanisms remains unknown.

It is notable that an energy-independent mechanism is also involved in glucose-mediated death resistance in cancer cells. Pyruvate not only serves as a link between glycolysis and mitochondrial respiration but also acts as a scavenger for oxidative free radicals through a non-enzymatic reaction[147]. Our recent studies have shown that pyruvate (uncoupled to ATP) plays a distinct role in promoting cancer cell survival[19]. Glycolytic pyruvate prevented RIPK1/3-dependent necroptosis caused by hypoxic stress in colon cancer cells through the suppression of mitochondrial free radicals[19]. Collectively, the manipulation of glucose uptake and metabolic pathways in conjunction with the application of cytotoxic drugs may promote anticancer effects and overcome chemoresistance (Figure 2).

With an increasing body of evidence suggesting that chemoresistance in colon cancers can be mediated by glucose, it is notable that dietary glutamine supplementation was shown to reduce tumor burden by increasing apoptosis and decreasing proliferation in the colons of mouse models that were submitted to colitis-associated cancer induction[148]. Although a glutamine-dependent chemopreventive effect on tumorigenesis was suggested to result from its anti-inflammatory activity, it remains unclear whether glutamine metabolism by first pass in epithelial cells (in place of glucose metabolism) may play a role in tumor suppression. The differential roles of glucose- and glutamine-dominant metabolism in the regulation of tumorigenesis warrant further investigation.

Bacterial LPS/Toll-like receptor 4-dependent apoptotic resistance in cancer

The innate immune receptor Toll-like receptor 4 (TLR4) forms a complex with CD14 and MD2 for sensing bacterial LPS. This receptor complex was originally identified on monocytes/macrophages and endothelial cells that are responsible for septic shock. Given the juxtaposition of commensal bacteria and intestinal mucosa, it had been assumed that normal gut epithelial cells were not equipped with LPS receptors. However, accumulating data showed that normal human colonocytes constitutively express CD14 in the absence of TLR4, and a strong immunoreactivity of CD14 and TLR4 was found in human colorectal carcinoma tissues, indicating a link between LPS/TLR4 signaling and tumor formation[149-151].

The activation of TLR4 signaling may contribute to tumorigenesis by promoting epithelial proliferation and/or by reducing cell death (Figure 2). *In vitro* studies in colon adenocarcinoma cell lines have demonstrated that LPS/TLR4 induced resistance to apoptosis *via* NFκB and ERK signaling, without modulating the rate of cell division[152,153]. Conversely, several studies have revealed enhancement of both cell *via*bility and proliferation following the activation of LPS/TLR4 in cancer cell lines[154,155]. Using chemically induced colitis-associated mouse cancer models, numerous studies have shown that the genetic absence of TLR4 and its downstream signaling molecules (*i.e.,* MyD88 and IKKβ) results in reduced tumor burdens[86,151,156]. A TLR4-dependent hyperproliferation of colonic epithelial cells was shown to be related to increased activation of COX/PGE2 and EGFR signaling in the mouse models of colon cancer[49,151,157].

Recently, we demonstrated that the abnormally upregulated TLR4 protein plays an anti-apoptotic role against its co-receptor CD14 during the development of colon cancer[158]. Stimulation with LPS induced CD14-mediated lipid signaling and led to colonic epithelial cell apoptosis, whereas TLR4 antagonistically promoted cell survival and cancer progression[29,158]. Our results showed that that dysfunction of this CD14/TLR4 antagonism in the context of the cellular death and survival response may contribute to the carcinogenic transformation of normal epithelial cells[158]. Finally, intracolonic administration of a TLR4 antagonist was shown to result in increased cancer cell apoptosis and reduced tumor burdern, suggesting the therapeutic potential of TLR4 blockade[158].

CONCLUSION

Greater understanding of the pathophysiological anti-death mechanisms that are present in cancer cells would enable advanced therapeutic interventions that could overcome chemoresistance. One of the key challenges of successful clinical translation is the ability to destroy tumors while sparing normal cells. By understanding the similarity between epithelial cytoprotection and cancer resistance, we may identify pathways that differ in normal and tumor cells for pharmacological manipulation. So far, abnormally expressed glucose transporters and TLR4, which are involved in both anti-death and pro-proliferative mechanisms, show great therapeutic potential. Drugs that target apoptotic machinery and signaling pathways, metabolic enzymes, and glucose transport are currently being investigated in clinical trials[159]. Agents to eliminate cancer stem cells, the cell type most resistant to death, are also in development[160]. In sum, targeting death resistance pathways may offer a promising therapeutic approach in addition to strategies such as inhibiting proliferation and angiogenesis and would offer hope to patients with refractory tumors.

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REVIEW

Treatment-related gastrointestinal toxicities and advanced colorectal or pancreatic cancer: A critical update

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Abstract

Gastrointestinal toxicities (GIT), including oral mucositis, nausea and vomiting, and diarrhea, are common side effects of chemotherapy and targeted agents in patients with advanced colorectal cancer and pancreatic cancer. Being often underreported, it is still difficult to precisely establish their burden in terms of both patient's quality of life and cancer care costs. Moreover, with the use of more intensive upfront combination regimens, the frequency of these toxicities is rapidly growing with a potential negative effect also on patient's outcome, as a result of dose reductions, delays or even discontinuation of active treatments. Thus, identifying patients at higher risk of developing GIT as well as an optimal management are paramount in order to improve patient's compliance and outcome. After the description of the main treatment-induced GIT, we discuss the current knowledge on the pathophysiology of these side effects and comment the scales commonly used to assess and grade them. We then provide a critical update on GIT incidence based on the results of key randomized trials conducted in patients with metastatic colorectal cancer and advanced pancreatic cancer.

Key words: Gastrointestinal toxicities; Oral mucositis; Diarrhea; Colorectal cancer; Pancreatic cancer

Core tip: Although extremely frequent, treatment-related gastrointestinal toxicities in patients with advanced colorectal cancer and pancreatic cancer are often underreported. As such, it is difficult to establish to what extent such toxicities affect both patient quality of life and cancer care costs. In our work we describe the main gastrointestinal toxicities as well as their pathophysiology and grading scales. Finally, based on the results of the main randomized clinical trials, we provide a critical update on their incidence with both chemotherapeutic agents and novel targeted drugs.

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INTRODUCTION

Gastrointestinal toxicities (GIT), including oral mucositis, nausea, vomiting, dyspepsia, diarrhea, and constipation are common adverse events of antineoplastic treatments. These side effects are frequently associated with classical chemotherapy drugs, although their rate of occurrence may vary according to treatment schedule[1]. Of all toxicities associated with cancer therapy, from a patient's perspective GIT are the most bothersome and consistently challenge patients' ability to tolerate cancer care[2].

Overall, the incidence of GIT is rising with the introduction of novel drugs and the adoption of more intense association regimens that combine polichemotherapy with targeted agents. At the same time, the vision of the gastrointestinal tract has markedly changed. The alimentary tube is no longer considered a compartmentalized anatomic tract divided in oral, gastric, small bowel and large bowel segments. Rather, it is now studied as an anatomic continuous in which the underpinning pathobiological phenomena such as mRNA TNF expression[3] may lead or contribute to concurrently emerging disturbances in different sites[4]. This concept is in line with the current approach in which regimen-related toxicities do not occur as solitary events, but present as cluster and may be holistically integrated with other common pathological pathways[5].

The assessment of GIT is largely dependent on clinician assignment of a grade based on a range of criteria established by various instruments. For describing toxicities (adverse events) associated with particular drug or radiation therapy regimens, the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE)[6] is probably the most commonly used. The periodic modification of CTC (now in its fourth version) makes longitudinal comparisons of studies difficult, as the criteria used to delineate severity scores have been inconsistent. A number of other grading systems have been used, often for specific components of GIT, with varying success. Aside from describing the toxicities of particular treatment regimens, scoring instruments play critical roles as research tools to assess the efficacy of toxicity interventions and as clinical guides for nursing interventions[7]. Finding a scoring instrument, which is easy to use and replicate, has clinical meaningfulness and is easily understood by all users has been challenging.

Notably, the health and economic burden of GIT associated with cancer treatments is significant[8]. Often treatment-related GIT toxicities result in unplanned medical consultation, emergency room visits, support infusion, gastrostomy placement and hospitalization leading to increased resource use and cost[9,10]. Furthermore, since management options for a number of GITs is limited, these toxicities may necessitate cancer treatment dose reduction or discontinuation thereby limiting optimum tumor control. In this review we summarize the scope of distinctive oral and gastrointestinal side effects of both standard chemotherapy regimens and novel agents used in colorectal and pancreatic cancers.

GIT IN ADVANCED COLORECTAL CANCER PATIENTS

Colorectal cancer (CRC) is the third most frequent cancer in men, after lung and prostate cancer, and is the second most frequent cancer in women after breast cancer. It accounts for 8% of new cancer cases in the United States, and is responsible for 8% to 9% of the estimated cancer deaths in the United States in 2014[11]. The therapeutic options available for the treatment of metastatic CRC have significantly increased over the last decade. Together with advances in surgical techniques, combination therapy of irinotecan and oxaliplatin with 5-fluorouracil and the introduction of novel drugs targeting epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) or vascular endothelial growth factor (VEGF) (bevacizumab, aflibercept, regorafenib, and ramucirumab) have led to a median survival times now approaching 30 mo[12-15]. Furthermore, increasing numbers of trials testing novel drugs have all expanded the treatment options. However, the addition of such targeted agents to standard regimens has often led to increased rates of gastrointestinal side effects.

GIT associated with standard cancer therapy regimens are very common and often clinically significant, though varying in severity[16,17]. Moreover, it is well known that treatment-related side effects involving the gastrointestinal tube such as oral mucositis and dysgeusia, nausea and vomiting, and diarrhea may often occur together and share a similar biological etiology. Mucositis is probably the most extensively studied toxicity of the gastrointestinal tract and it refers to cancer regimen-related mucosal damages, which can either occur in the oral cavity, *i.e.,* oral mucositis or stomatitis, or in lower regions of the gastrointestinal tract. The usual presentation of oral mucositis includes erythema and/or ulceration of the mucosa, whereas gastrointestinal mucositis usually presents with pain, bloating, diarrhea, nausea and vomiting. As a result, mucositis is associated with considerable morbidity, diminished quality of life as

well as negative health and economic outcomes[18-20].

Similar to many other cancer treatment-related toxicities, GIT tends to be reported only when severe cases occur. Consequently, the incidence of this side effect is largely underestimated and often inconsistent, ranging from as low as 30% to almost 100% when all grades of mucositis are considered. Therefore, identification of subjects at higher risk as well as optimal management of this side effect can lead to better treatment tolerance, improved quality of life and more appropriate resource allocation. Mucositis risk depends on therapy-related factors such as the type of cancer drug administered, the regimen used, its dosage and schedule, and patient-related factors such as gender, age, baseline comorbidities and tumor diagnosis. Interestingly, also genomic plays a relevant role in the risk of developing oral mucositis[21,22], as well as the disruption of composition and function of the host-microbiota local environment[23,24].

Amongst the chemotherapeutic agents commonly used for patients with metastatic CRC, 5-FU and irinotecan are the two drugs with the highest risk for oral and gastrointestinal mucositis, respectively. In addition, multiple cycles of chemotherapy also seem to play an important role as risk factor for oral mucositis, mainly as a result of a cumulative effect, and the risk for GIT may increase when more intensive treatments such as FOLFOXIRI are used. Female gender results associated with higher risk of developing severe mucositis, as suggested by the results of clinical trials conducted in CRC patients treated with 5-fluorouracil[7,25,26].

Genomic determinants of GIT are associated with genes governing both drug metabolism [pharmacokinetic (PK)] and its pathobiology. An example of a PK-related pharmacogenomic marker of toxicity risk is the catabolic enzyme, dihydropyrimidine dehydrogenase (DPD) that plays a critical role in 5-FU metabolism. Insufficient DPD activity results in toxic levels of 5-FU and is associated with increases in both hematologic and non-hematologic toxicities[27]. DPD activity is affected by at least two variants of the DPYD gene, DPYD*2A and D949V. Additional DPYD variants have been uniquely described in African Americans[28,29]. As a consequence, a FDA-approved test to assess DPD activity is commercially available.

Improved understanding of the pathogenesis of GIT has led to opportunities to assess its variable risk among patients. For example, oxidative stress, which is responsible of reactive oxygen species (ROS) formation, typically occurs after the administration of chemotherapy and eventually leads to tissue damage. Preclinical studies showed that changes in the expression of genes of with single-nucleotide polymorphisms involved in the metabolism of reactive oxygen species were associated with increased risk of mucositis[7,30].

The pathogenesis of GIT is a complex process involving five different predictable phases, which is usually initiated by direct cell damage from chemotherapy. DNA damage, ROS formation and the subsequent death of the basal epithelial cells lead to the release of endogenous damage-associated pattern molecules (CRAMPs) which, in turn, trigger the innate immune response[31] as well as several other pathways involved in the production of pro-inflammatory cytokines. The nuclear factor Kappa-B (NF-κB) pathway is probably one of the most extensively investigated[32,33] and its activation eventually translates into signal amplification with local recruitment of inflammatory cells[34]. Development of symptomatic deep ulcerations, which can be easily colonized by oral bacteria, may lead to an extension of the mucosal damage itself. Healing usually occurs in the last stage with a complete restitutio ad integrum.

Similar to oral mucositis, gastrointestinal mucositis is a complex process, a result of both direct and indirect injury leading to crypt cell death, breakdown of the mucosal barrier and lastly to mucosal inflammation. Rapidly dividing cells are particularly sensitive to many cytotoxic chemotherapeutic agents, thus the GI tract is extremely vulnerable. The first abnormality detected in human small intestine on day 1 after chemotherapy is an increase of the rate of cells switching to apoptosis. Such phenomenon is then followed by a reduction in crypt length as well as villus area and mitotic index, reaching a nadir on day 3. Interestingly, the rate in apoptosis not always correlates with the severity of mucositis. Finally, on day 5 after chemotherapy a rebound hyperplasia usually leads to a gradual normalization of the tissue and to re-epithelialization[18].

Chemotherapy-induced diarrhea is most commonly reported with fluoropyrimidines and irinotecan, and this potentially dangerous side effect often needs to be aggressively managed[35]. Both drugs can cause acute damage of the intestinal mucosa leading to loss of epithelium. As a result, the increased amount of fluids that transits from the small bowel to the colon exceeds the absorptive capacity of the colon, finally resulting in diarrhea[36,37]. Moreover, while delayed-onset irinotecan-associated diarrhea appears to be multifactorial with both cytokine and direct toxic inflammatory-mediated effects on the intestinal mucosa as well as an alteration of the motility[38], the early-onset diarrhea is cholinergically mediated. Occurring in 45%-50% of patients, during or within several hours of drug infusion, such diarrhea seems to be caused by the structural similarity of the drug with acetylcholine. Moreover it is often accompanied by other symptoms of cholinergic excess such as abdominal cramping, rhinitis, lacrimation and salivation[37]. A number of clinical studies have demonstrated the role of UGT1A1 genotyping as a potential marker for CPT-11 toxicity[39], which may also correlate with severe hematological toxicity[40]. Once again, CRC patients exposed to multiple chemotherapy cycles may be at higher risk

for chemotherapy-induced diarrhea[41]. Obviously, the potential of the primary tumor to contribute to GI symptoms cannot be overlooked.

Little evidence is currently available on chemotherapy-induced esophageal mucositis mainly because most of the symptoms associated with esophageal mucositis are usually attributed to gastroesophageal reflux disease or to both viral and fungal infections. However, the effect of chemotherapy on esophageal epithelium has been described before and it appears that chemotherapeutic agents damage the dividing and differentiating cells, leading to a thin and ulcerated epithelium[42]. Similarly, modest information exists about mucositis of the stomach. Overall, gastrointestinal mucositis can be debilitating and in some cases also life-threatening: as a matter of fact, volume depletion can lead to acute renal failure, electrolyte disorders and metabolic acidosis.

With both irinotecan and oxaliplatin classified as moderate emetogenic cytotoxic drugs, nausea and vomiting can also be a relevant issue in patients with CRC treated with such agents[43]. Chemotherapy-induced vomiting and nausea can greatly affect patient's quality of life with subsequent poor compliance to chemotherapy. As a matter of fact, such symptoms not only can lead to metabolic disorders, anorexia and decline of the patient's performance status, but they can also be responsible for discontinuing potentially useful anticancer treatments[44,45]. Similarly to oral mucositis, the risk of nausea and vomiting depends on various factors including the type of drug administered, its dosage, schedule and route of administration as well as the patient's age, sex and his/her past medical history[44-46]. The role of pharmacogenomics in the occurrence and intensity of nausea and vomiting has not been fully unrevealed and deserves further studies.

Vomiting is a result of a multistep pathway which is controlled by the brain and is usually triggered by afferent impulses to the vomiting center, located in the medulla, originating from the chemoreceptor trigger zone, pharynx and gastrointestinal tract (*via* vagal afferent fibers) and cerebral cortex. Once the vomiting center is adequately stimulated, efferent impulses are sent to the salivation center, abdominal muscles, respiratory center and cranial nerves and vomiting occurs[47-49]. Chemotherapeutic agents as well as their metabolites usually lead to the activation of neurotransmitter receptors located in the chemoreceptor trigger zone, vomiting center and GI tract. The main neuroreceptors involved in the emesis are the serotonin (5-hydroxytryptamine) and dopamine receptors, which are targeted by many antiemetic agents[49]. Nausea and/or vomiting induced by chemotherapy are usually classified as acute, delayed, anticipatory, breakthrough or refractory. The timing of occurrence is the main difference between the acute-onset and the delayed-onset with nausea and/or vomiting occurring before and after 24 h from the administration of the drug, respectively. Anticipatory nausea and/or emesis occurs before patients receive chemotherapy and is usually associated with a negative past experience with chemotherapy. Finally, breakthrough emesis refers to vomiting that occurs despite prophylactic treatment. The frequency of chemotherapy-induced vomiting, as mentioned before, depends on many factors but primarily on the ematogenic potential of the specific chemotherapeutic agents. The most recent MASCC and ESMO treatment guidelines follow the Grunberg classification for intravenous agents, which defines 4 different risk levels of vomiting: high emetic risk where 90% or more of patients experience acute emesis, moderate emetic risk, 30% to 90% of patients experience acute emesis, low emetic risk, 10% to 30% of patients with acute emesis and minimal emetic risk with fewer than 10% of patients experiencing acute emesis[50].

Rectal cancer patients who undergo chemoradiation may also suffer from proctitis, a treatment-induced proctopathy consisting in a painful epithelial damage to the rectum, usually associated with minimal or no inflammation. Based on the timing of symptoms, radiation proctitis can be classified as acute if it occurs during or within six weeks of radiation therapy or as chronic if it has a more delayed onset[51]. Risk factors include the dose of radiation, area of exposure, method of delivery as well as patient-related factors such as inflammatory bowel disease. Once again, specific polymorphisms of genes involved in the disease pathogenesis may be associated with greater risks for toxicity. For example, VEGFR2 H427Q QQ genotype was significantly associated with increased severe upper gastrointestinal tract mucositis[52].

The general pathobiology for proctitis is similar to other stratified squamous mucosa. While acute proctitis is a consequence of the direct mucosa damage form radiation exposure, chronic proctitis results from progressive epithelial atrophy and fibrosis associated with chronic mucosal ischemia and obliterative endarteritis. The main symptoms of acute radiation proctitis include diarrhea, tenesmus, urgency and mucus discharge; severe bleeding is usually more common in chronic proctitis. Occasionally patients may also develop symptoms of obstructed defecation due to strictures such as constipation and rectal pain.

Finally, another common gastrointestinal toxicity is dysgeusia; transient alteration in taste often leads to reduced appetite as well as low energy intake and weight loss. The chemotherapeutic agents that have been most associated with taste alterations include irinotecan, oxaliplatin, fluorouracil and gemcitabine[53]. These drugs may affect taste by stimulating taste receptors particularly when they are secreted in saliva. Dysgeusia often persists after drug clearance due to damage to the taste buds.

Clinical assessment of GIT

A number of scales are available to assess the severity of GIT. In general, toxicity of each area of the GI

Table 1 Oral mucositis grading scales

CTCAE version 4.03	Grade				
	1	2	3	4	5
Description	Asymptomatic or mild symptoms; intervention not indicated	Moderate pain; not interfering with oral intake; modified diet indicated	Severe pain; interfering with oral intake	Life-threatening consequences; urgent intervention indicated	Death
WHO			**Grade**		
	0 (none)	Ⅰ (mild)	Ⅱ (moderate)	Ⅲ (severe)	Ⅳ (life-threatening)
Description	None	Oral soreness, erythema	Oral erythema, ulcers, solid diet tolerated	Oral ulcers, liquid diet only	Oral alimentation impossible

CTCAE: Common Terminology Criteria for Adverse Events version; WHO: World Health Organization.

tract is graded independently. For oral mucositis the World Health Organization (WHO) scale (Table 1) or the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) (Table 1) are among the most frequently used. While NCI-CTCv4 is limited to pain and ability to eat, the WHO scale combines both functional and objective (erythema and ulceration) assessments and probably provides a more complete indication of the severity of the condition. Gastrointestinal mucositis as well as nausea and vomiting are usually graded based on CTCAE scale. The severity of chemotherapy-induced diarrhea for instance, is based on the number of stools per day or increase in stoma output compared to baseline, as well as on the need for hospitalization and the effect on self-care activities. Similarly, vomiting is graded from 0 to 5 based on number of episodes per day and the need for hospitalization or total parental nutrition.

GIT associated with specific treatment regimens

The current management of metastatic CRC involves various active drugs, given either in upfront combination or as single agents in later treatment lines. Although curative rates remain low for patients with advanced disease, the median overall survival has dramatically improved with modern treatments. Upfront 5FU-based doublet regimens with irinotecan or oxaliplatin combined with bevacizumab are currently widely used, and these combinations are usually associated with significantly increased rates of gastrointestinal mucositis compared to those previously reported (Table 2). Recently, the Italian phase 3 TRIBE study has randomized untreated patients with metastatic CRC to receive either FOLFOXIRI in combination with bevacizumab or FOLFIRI plus bevacizumab[13], with significant overall survival improvement for patients enrolled in the experimental arm (31 mo *vs* 25.8 mo, HR = 0.79). It came as no surprise, however, that the better outcome results were associated with increased toxicity as the overall safety profile, mainly in terms of GIT, was significantly worse for the triplet compared with the FOLFIRI plus bevacizumab arm. Severe or life-threatening grades of diarrhea were reported in 10.6% of patients enrolled in the FOLFIRI plus bevacizumab arm and in 18.8% of those randomized to the FOLFOXIRI plus bevacizumab arm (P = 0.01). Similarly, stomatitis was described in 8.8% of patients treated with the triplet *vs* 4.3% (P = 0.048).

Results of the randomized CRYSTAL study showed that the upfront addition of cetuximab to FOLFIRI improved median OS of patients whose tumors did not have mutations at KRAS codons 12 and 13[54,55]. In KRAS wild-type patients, median PFS was 9.9 mo for those exposed to cetuximab *vs* 8.7 mo in those receiving FOLFIRI alone (HR = 0.69, 95%CI: 0.56-0.97, P = 0.012). Median OS was also significantly improved in the arm containing cetuximab (23.5 mo *vs* 20.0 mo, HR = 0.79, 95%CI: 0.67-0.94, P = 0.009). As expected, the safety profile showed a 50% increase of the frequency of grade 3 and grade 4 diarrhea which occurred in 15.7% of the patients allocated to the cetuximab arm *vs* 10.5% of those enrolled in the standard arm. Similarly, the PRIME trial compared the combination of FOLFOX plus panitumumab with FOLFOX alone, in patients with metastatic CRC who did not receive any prior treatment[56]. The study met its primary endpoint (PFS) in the KRAS wild-type population, with a median PFS for FOLFOX combined to panitumumab of 9.6 mo *vs* 8.0 mo for the FOLFOX alone arm (HR = 0.80, 95%CI: 0.66-0.97, P = 0.02). The addition of the monoclonal antibody, however, resulted in a significantly increased rate of diarrhea (18% *vs* 9%) and mucositis (9% *vs* < 1%).

FIRE 3 and CALGB 80405 were designed to assess whether cetuximab or bevacizumab was a more effective partner for doublet chemotherapy in the first-line treatment in patients with KRAS exon 2 wild-type metastatic CRC. In the European FIRE-3trial, patients were randomly assigned to receive FOLFIRI plus cetuximab or FOLFIRI plus bevacizumab. Although no differences were noted in terms of response or PFS, median OS was significantly longer in the FOLFIRI plus cetuximab group (HR = 0.77, P = 0.017)[12]. By contrast, the US-based randomized phase 3 trial CALGB 80405, which compared first-line cetuximab or bevacizumab in combination with FOLFOX or FOLFIRI, failed to show a difference in terms of survival between

Table 2 Frequency of gastrointestinal toxicities in metastatic colorectal cancer: results from main recent clinical trials

Trials	Nausea		Vomiting		Diarrhea		Oral mucositis	
	Any grade	G3-G4	Any grade	G3-G4	Any grade	G3-G4	Any grade	G3-G4
Folfiri + cetuximab (FIRE-3 trial, Heinemann *et al*[12]. *Lancet Oncol* 2014)	45%	3%	22%	2%	46%	11%	38%	4%
Folfiri + bevacizumab (FIRE-3 trial, Heinemann *et al*[12]. *Lancet Oncol* 2014)	58%	5%	29%	3%	49%	12%	41%	3%
Folfiri + aflibercept (VELOUR trial, Van Cutsem *et al*[60]. *J Clin Oncol* 2012)	53.4%	1.8%	32.9%	2.8%	69.2%	19.3%	54.8%	13.8%
Regorafenib (CORRECT trial, Grothey *et al*[63]. *Lancet* 2013)	14%	< 1%	8%	1%	34%	7%	27%	3%
Folfoxiri + bevacizumab (Tribe trial, Loupakis *et al*[13]. *N Engl J Med* 2014)		2.8%		4.4%		18.8%		8.8%
Folfox + bevacizumab (Tribe trial, Loupakis *et al*[13]. *N Engl J Med* 2014)		3.2%		3.2%		10.6%		4.3%

the two targeted agents[57]. Both FIRE-3 and CALGB 80405 trial, however, showed a similar safety profile. The most common grade 3 or 4 adverse events in both treatment groups were diarrhea (11% of patients in the cetuximab arm and 14% in the bevacizumab arm for FIRE-3 trial, 11% of patients in the cetuximab arm and 8% in the bevacizumab arm for CALGB 80405). The frequency of stomatitis as well as nausea and vomiting was in line with previously reported results.

Even after protocol amendment because of safety-concerns, very high rates of severe diarrhea (35% of grade 3-4) were reported when the triplet regimen FOLFOXIRI was associated with panitumumab in 37 molecularly selected CRC patients enrolled in the TRIP study[58]. Accordingly, in the POCHER trial that exposed 42 unresectable metastatic CRC patients to cetuximab plus a chronomodulated combination of 5-Fluorouracil, oxaliplatin and irinotecan, grade 3 and 4 diarrhea occurred in 93% and 36% of patient before and after dose reduction[59].

Second-line chemotherapy also includes novel agents. Aflibercept, a multitarget antiangiogenic fusion protein, was combined to FOLFIRI in the multinational phase Ⅲ trial VELOUR, showing significantly prolonged OS and PFS in pretreated advanced CRC patients compared with chemotherapy alone[60]. More grade 3 or 4 adverse events were reported in the aflibercept arm compared with the placebo arm. In particular, higher rates of severe diarrhea (19.3% *vs* 7.8%) and stomatitis/ulceration (13.7% *vs* 5%) were noted. Furthermore, diarrhea was one of the toxicities that most frequently led to chemotherapy discontinuation in the experimental arm. Ramucirumab is a novel VEGFR2 inhibitor already approved in pretreated patients with advanced gastric cancer[61]. The RAISE study compared FOLFIRI plus ramucirumab to FOLFIRI plus placebo in 1072 CRC patients who had failed first-line chemotherapy[62]. The trial met its primary endpoint showing a 2 mo increase in median OS (HR = 0.844, 95%CI: 0.73-0.97, P = 0.022). The combination of FOLFIRI and ramucirumab however was associated with higher incidence of any grade stomatitis (30.8% *vs* 20.8%) and diarrhea (59.7% *vs* 51.5%) compared to FOLFIRI alone; of note, the rate of severe cases was not statistically different between treatment arms.

Regorafenib is a small tyrosine kinase inhibitor which has been approved in pretreated patients with advanced CRC based on the positive survival results of the double-bind, placebo-controlled phase Ⅲ trial CORRECT trial[63]. The most common adverse events included GI toxicities of any grade, such as diarrhea (34% *vs* 8% in placebo arm), oral mucositis (27% *vs* 4%), nausea (14% *vs* 11%), constipation (8% *vs* 5%), and vomiting (8% *vs* 5%); furthermore diarrhea was also one of the most frequent regorafenib-related grade 3 or grade 4 adverse events. Since the combination of regorafenib and cetuximab could be a valuable strategy to overcome acquired resistance to EGFR-inhibitors[64], the gastrointestinal toxicity profile of the combination deserves to be further studied.

Finally, a number of MEK inhibitors have progressed into clinical trials and are currently under evaluation. The mitogen-activated protein kinase (MAPK) signaling pathways involve a family of protein kinase which play critical roles in regulation of many cellular activities such as cell proliferation, survival, differentiation and angiogenesis. MAPK pathway blockade through MEK inhibition can be an effective approach in patients with metastatic CRC. Amongst the MEK inhibitors currently under development, trametinib (GSK1120212), a potent small molecule inhibitor of MEK kinase, is the most extensively investigated. An early phase Ⅰ trial of trametinib enrolled patients with advanced solid tumors, including patients with chemotherapy refractory advanced colorectal cancer. Dose-limiting toxicities included diarrhea[65].

In this area, the study of patients' immune genetics and inflammation to predict the risk of increased gastrointestinal toxicity has been suggested, but not fully elucidated[66,67].

GIT IN PATIENTS WITH ADVANCED PANCREATIC ADENOCARCINOMAS OR NEUROENDOCRINE CANCER

Pancreatic cancer is one of the deadliest among the solid malignancies, and pancreatic ductal adenocarcinoma (PDAC) accounts for over 95% of all cases diagnosed[68]. Most patients present with metastatic disease at the time of diagnosis and the goal of treatment is therefore palliative. Historically, gemcitabine was the standard of care for first-line treatment, since randomized studies combining gemcitabine with platinum, erlotinib[69] or capecitabine[70] only produced marginal clinical improvements. Recently, the French phase Ⅲ trial PRODIGE 4/ACCORD 11 showed that upfront FOLFIRINOX was superior to gemcitabine in patients with advanced pancreatic cancer, in terms of median OS (11.1 mo *vs* 6.8 mo, HR = 0.57), median PFS (6.4 mo *vs* 3.3 mo), and objective responses[71]. In the study, however, treatment-related GIT were significantly greater with FOLFIRINOX compared to gemcitabine group, mainly because of a higher incidence of grade 3 or 4 vomiting (14.5% *vs* 4.7%, P = 0.002) and diarrhea (12.7% *vs* 1.2%, P = 0.0001). While a three-drug antiemetic regimen is suggested to provide optimal control of nausea and vomiting[72], retrospective studies reassuringly suggest that a more conservative de-intensified schedule of the same triple regimen may be equally effective and less toxic[73]. The phase Ⅲ MPACT trial set the combination of gemcitabine plus nab-paclitaxel as a novel standard treatment option showing its superiority in terms of response rate, median PFS, and median OS compared to gemcitabine alone[74]. The safety analysis of the trial found that the combination was fairly tolerable, and although it was associated with more side effects than gemcitabine alone, the overall quality of life was improved[75]. Notably, the combination produced higher incidence of any grade diarrhea compared to gemcitabine (37% *vs* 13%) as well as more severe diarrhea (6% *vs* 1%)[76].

The development of novel anticancer agents, interfering with tumor's microenvironment or with the tumor cell itself, is also producing advances[77]. A randomized phase Ⅱ trial with gemcitabine and TH-302 (evofosfamide), a hypoxia-activated prodrug, showed potential therapeutic efficacy, increasing PFS by 2 mo[78]. Enrolled patients were randomized to gemcitabine alone or gemcitabine plus TH-302 at two different doses of 240 mg/m^2 or 340 mg/m^2. The combination regimen produced increased skin, mucosal and hematological toxicities. In particular, a higher incidence of all-grade stomatitis was reported for the combination compared to gemcitabine alone (18% for the lower TH-302 dose, 36% for the higher TH-302 dose, 7% for gemcitabine alone) although the cases of severe stomatitis were numerically similar among the treatment arms. MAESTRO, a phase Ⅲ randomized trial in which patients are randomized to gemcitabine alone versus gemcitabine plus TH-302 at the dose of 340 mg/m^2 is currently ongoing. The combination of gemcitabine and masitinib also produced interesting clinical results in a recent phase Ⅲ trial, although increased rates of nausea (58% *vs* 47%, P = 0.036) and vomiting (50% *vs* 37%, P < 0.001) were noted for the experimental arm[79].

Neuroendocrine tumors (PNET) represent approximately 2% of all pancreatic cancers. Due to their rarity and heterogeneity, the advances in their characterization and treatment have been slow, and a limited number of efficacious systemic treatments are currently available[80]. Large phase Ⅲ clinical trials have demonstrated that everolimus and sunitinib could significantly improve PFS in these patients. Everolimus belongs to mammalian target of rapamycin (mTOR) inhibitor. Aberrant signaling through the mechanistic mTOR pathway has been implicated in neuroendocrine tumorigenesis, and altered expression of mTOR pathway components has been observed in NETs. A randomized placebo-controlled phase Ⅲ study of patients with PNET demonstrated a significantly improved PFS with everolimus (11.0 mo *vs* 4.6 mo, HR = 0.35, 95%CI: 0.27-0.45)[81]. Among drug-related adverse events oral stomatitis, rash, diarrhea, and fatigue should be included. Aphthous-like oral stomatitis has been identified as one of the most common dose-limiting toxicities associated with the drug[82], and the pathogenesis of this side effect has been demonstrated peculiar[83]. GI toxicities are frequent, including stomatitis, diarrhea and vomiting, with most of them being grade 1 or 2, though some cases of stomatitis and diarrhea were grade 3 or grade 4.

Sunitinib is an oral multitarget tyrosine kinase inhibitor with antiangiogenic properties. Compared to placebo, sunitinib doubled median PFS from 5.5 to 11.4 mo when given continuously at the dose of 37.5 mg/d to patients with well-differentiated pancreatic NET enrolled in a multinational, randomized, double-blind, placebo-controlled phase 3 trial[84].

The GI toxicities associated with sunitinib were diarrhea, nausea, vomiting, dysgeusia and stomatitis; the majority of adverse events was grade 1 or 2 and easily managed wit appropriate medical therapy[85].

A comprehensive description or major GI toxicities of the above cited drugs are represented in Table 3.

CONCLUSION

Chemotherapy-induced gastrointestinal toxicities not only are a common problem in cancer patients but they often are clinically significant. Defining the epidemiology of these peculiar toxicities has always been compelling for many reasons including underreporting and differences in assessment techniques and scales. Overall, they remain a significant burden for patients undergoing systemic

Table 3 Frequency of gastrointestinal toxicities in advanced pancreatic cancer: results from main clinical trials

Trials	Nausea		Vomiting		Diarrhea		Oral mucositis	
	Any grade	G3-G4	Any grade	G3-G4	Any grade	G3-G4	Any grade	G3-G4
Gemcitabine + TH-302 (240 mg/m^2) (Borad *et al*[78]. *J Clin Oncol* 2014)	39%	10%	24%	6%	28%	3%	18%	0%
Gemcitabine + TH-302 (340 mg/m^2) (Borad *et al*[78]. *J Clin Oncol* 2014)	46%	5%	38%	8%	36%	4%	36%	0%
Sunitinib (Yao *et al*[81]. *N Engl J Med* 2011)	45%	1%	34%	0%	59%	5%	22%	4%
Everolimus (Raymond *et al*[84]. *N Engl J Med* 2011)			15%	0%	34%	3%	64%	7%
Folfirinox (PRODIGE 4/ACCORD11 trial, Conroy *et al*[71]. *N Engl J Med* 2011)				14.5%		12.7%		
Nab-paclitaxel + gemcitabine (MPACT trial, Von Hoff *et al*[74]. *N Engl J Med* 2013)						6%		

chemotherapy with or without targeted drugs, with potentially negative effects on both patient's outcome and cancer care costs. Moreover, the more aggressive upfront regimens often used nowadays in patients with metastatic colorectal cancer together with the introduction of novel targeted therapies are likely to worsen the issue. Improved understanding of the pathophysiology underlying gastrointestinal toxicities has allowed identifying patients at higher risk, developing new effective treatments to prevent or help the recovery from such disturbances and to provide symptomatic relief. Still, management of chemotherapy-induced gastrointestinal toxicities remains a major challenge with future studies needed in order to identify subjects who are genetically predisposed to develop severe GI side effects.

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REVIEW

Laparoscopic sleeve gastrectomy: More than a restrictive bariatric surgery procedure?

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Abstract

Sleeve gastrectomy (SG) is a restrictive bariatric surgery technique that was first used as part of restrictive horizontal gastrectomy in the original Scopinaro type biliopancreatic diversion. Its good results as a single technique have led to a rise in its use, and it is currently the second most performed technique worldwide. SG achieves clearly better results than other restrictive techniques and is comparable in some aspects to the Roux-en-Y gastric bypass, the current gold standard in bariatric surgery. These benefits have been associated with different pathophysiologic mechanisms unrelated to weight loss such as increased gastric emptying and intestinal transit, and activation of hormonal mechanisms such as increased GLP-1 hormone and decreased ghrelin. The aim of this review was to highlight the salient aspects of SG regarding its historical evolution, pathophysiologic mechanisms, main results, clinical applications and perioperative complications.

Key words: Bariatric surgery; Sleeve gastrectomy; Severe obesity; Dyslipidemia; Hypertension; Type 2 diabetes mellitus

Core tip: The most salient aspects of sleeve gastrectomy, a restrictive bariatric surgery technique yielding better results than other restrictive techniques that cannot simply be explained by weight loss, are reviewed.

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HISTORY: FROM OUTSET TO TODAY

Sleeve gastrectomy (SG) began to be used in 1988 as a variation of biliopancreatic diversion (BPD) with duodenal switch[1-3]. In contrast to the BPD described by Scopinaro *et al*[4] in which a horizontal gastrectomy was performed, the pylorus and duodenum were preserved in SG, yielding a reduction in dumping symptoms and marginal ulcers. In addition, gastrectomy was more restrictive, permitting a decline in the malabsorptive component and nutritional secondary effects[2]. Initially, this technique was performed openly, with Ren *et al*[5] being the first to perform it laparoscopically in the late 1990's. In the early 2000's, given the high frequency of complications in patients with a high body mass index (BMI)[6], Regan *et al*[7] described a two-step approach to treat patients with high surgical risk. In a first step, SG was implemented to achieve sufficient weight loss to permit the Roux-en-Y gastric bypass (RYGB) or BPD to be performed more safely in a second step[8,9]. Given the good results obtained, a second intervention was unnecessary in many cases which, together with low morbidity and mortality, rapidly installed SG as a single procedure[10-12]. Subsequently, Baltasar *et al*[13] recommended a multipurpose strategy, applying SG as a single procedure in mildly-obese patients or after failed gastric banding, and as a 2-step procedure for high-risk patients, who were either extremely obese or had serious comorbidities. In recent years, some technical modifications, such as a progressive decrease in gastric remnant size, have been made in order to prevent weight gain in the long term[14], or the use of natural transluminal orifice endoscopic surgery[15] and single incision laparoscopic surgery[16].

SG has gradually gained in popularity, becoming established as the second most used bariatric procedure worldwide, closer to RYGB, the considered gold standard. Thus, according to the International Federation for the Surgery of Obesity and Metabolic Diseases, between 2008 and 2013, SG use increased from 5.3% to 27.9% of all procedures while RYGB, albeit remaining the most widely-used technique, has fallen from 49.0% to 46.6%[17].

THE TECHNIQUE

SG is a bariatric technique consisting of subtotal vertical gastrectomy with preservation of the pylorus, including longitudinal resection of fundus, corpus and antrum, to create a tubular duct along the lesser curvature. Resection comprises approximately 80% of the stomach and the remnant gastric has a capacity > 100 mL. It is considered an easier technique than other procedures such as RYGB, since multiple anastomoses are required[18] (Figure 1). Variants of SG have been described, and although no comparative studies have been conducted, none seems to offer advantages. Furthermore, SG has been performed with different degrees of intestinal bypass, including variants with 2 exits from the stomach such as SG transit with bipartition[19] and SG with loop bipartition[20]. In an attempt to achieve a surgery with more metabolic effects, SG has also been linked with ileal transposition[21]; finally, short-term studies on SG with a gastric band have been reported[22].

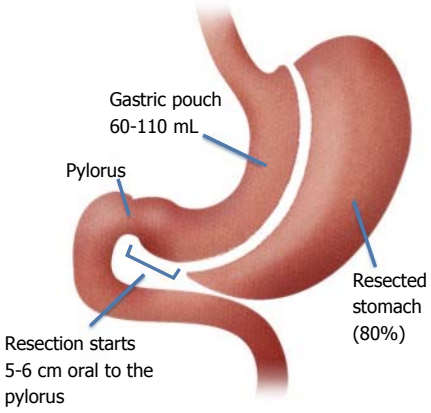

Figure 1 Sleeve gastrectomy.

MECHANISMS

SG yields better results than other restrictive techniques and is similar to RYGB in terms of weight loss and carbohydrate metabolism improvement in the short and medium term[23]. This SG superiority over other restrictive techniques has been related to different mechanisms such as modification of gastrointestinal motility, hormonal mechanisms, alterations in bile acids and gut microbiote.

Unlike other restrictive techniques such as gastric banding, SG provokes a rapid gastric emptying[24] and accelerated intestinal transit[25]. It seems that the rapid transit may trigger hormonal mechanisms that will be described below; it could also cause increased satiety, as occurs with drugs that enhance gastric emptying[26].

GLP-1 is an incretin hormone secreted by L-cells of the distal intestine in response to eating. It has beneficial effects on weight and glucose metabolism since it promotes insulin secretion, inhibits gastric emptying, glucagon secretion and hepatic glucose production[27]. SG has repeatedly produced an exaggerated postprandial increase in GLP-1[28-30] comparable to that of RYGB. In the latter, the rise in GLP-1 could be explained by the hind-gut hypothesis,

in which stimulation of the distal gut caused by the bypass lead to an amplified increase in GLP-1. However, after SG, the mechanism by which the surgery would increase GLP-1 secretion is unclear. One hypothesis could be that the enhanced transit resulting from SG also causes distal intestine stimulation[24]. A further possibility would arise from the lack of gastric response to the intestinal signals that normally slow emptying[24]. Others have proposed that an increase in GLP-1 levels would be an effort to restore intestinal gastric motility in response to accelerated gastric emptying[31]. Since GLP-1 response is also increased by infusing nutrients directly into the duodenum, the existence of an independent gastric emptying mechanism has also been suggested[24]. Moreover, given the rapid increase in GLP-1 following ingestion and presumably before nutrients contact L-cells, the existence of a proximal-distal circuit causing GLP-1 secretion has been proposed that does not require direct contact between chime and L-cells, which could be mediated *via* a neural[32] or hormonal pathway through cholecystokinin (CCK)[33].

Peptide YY (PYY), also known as peptide tyrosine tyrosine or pancreatic peptide YY_{3-36}, is an anorexigenic peptide released by L-cells in mucosa of the gastrointestinal tract, especially the ileum and colon, in response to feeding[34]. In addition to reduce appetite, PYY increases nutrient absorption in the ileum, inhibits gastric and pancreatic secretion, attenuates gallbladder contraction and slows gastric emptying. Reduced secretion in obese patients, which is associated with lower satiety, has been reported[35]. Like GLP 1, numerous studies have demonstrated a significant increase in PYY after SG, and again the results are comparable to those observed after RYGB[29,30], suggesting that the mechanism for increase will be shared.

Ghrelin is a neuropeptide with orexigenic action predominantly synthesized by oxyntic cells of the gastric fundus[36]. Under physiologic conditions, ghrelin levels increase during fasting with a preprandial peak and are suppressed by food. It also has diabetogenic effects such as the suppression of insulin secretion[37]. A drop in ghrelin concentrations after SG compared to baseline levels[38,39] and other restrictive techniques[40,41] or RYGB[28,29] has consistently been demonstrated. This drop off has been associated with fundus resection and there is speculation that it may be one of the main mechanisms accounting for the superiority of SG over other restrictive techniques and its similarity to RYGB. Nevertheless, some experimental studies found that the decline in ghrelin concentrations could not be decisive. Chambers *et al*[42] showed that ghrelin-deficient mice continued to lose weight, had improved glucose metabolism and inappetence for fatty foods after SG. However, the authors warned that a possible compensatory mechanism in ghrelin-deficient animals may underestimate the effects of surgery. In favor of the beneficial effects of ghrelin reduction after SG, an increase in ghrelin after weight loss by diet or by other restrictive techniques has been observed[40,41]. This suggests that weight loss triggers compensatory mechanisms to recover weight that could be deleted after SG[43].

Leptin, synthesized in white adipose tissue proportionally to the amount of body fat[44], reduces intake and body weight through actions in the central nervous system. In obesity, a decreased sensitivity to leptin has been suggested, resulting in an inability to detect satiety despite high energy stores[45]. It is unclear whether the improvement in leptin resistance plays a direct role in weight loss after SG. While related genes seem to increase its expression[46], recent studies suggest that the reversal of leptin resistance could be regulated by protein availability[47].

Increasing endocrine functions for bile acids have been recognized and associated with an increased GLP-1 response, carbohydrate metabolism improvement and reducing liver steatosis[48]. The increased serum bile acid concentrations after SG[49] are probably related to rapid transit that will increase their availability in the area of maximum absorption, the terminal ileum. It also appears that these effects could be mediated by the farsenoid X receptor (FXR), since Ryan *et al*[50] showed that this pathway is needed to improve glucose metabolism, prevent compensatory hyperphagia and maintain long-term weight loss after SG.

Another mechanism which potentially influences the metabolic benefits of SG is the change in the gut microbiome, which improves the flora composition as in lean subjects[50,51] in a similar way to but less striking than RYGB. Although the mechanisms are yet to be discovered, the way is open to a complex and promising system of host-bacteria interactions[52].

With a view of greater perspective, control energy homeostasis involves a sophisticated communication system among the gut, adipose tissue and central nervous system[42,46]. *Via* hormonal and neural signals, the central nervous system integrates the information on what happens in the gut, *e.g.*, type and amount of ingested nutrients and on energy reserves and acts by regulating appetite, satiety and feeding behavior. For instance, against a negative energy balance, this system could compensate by hyperphagia or increasing preference for high-calorie food to restore normal weight[46]. In this respect, the key for the effectiveness of SG as metabolic surgery appears to lie in preventing these compensatory responses, modifying both hormonal and neural signaling pathways or even leading to changes at central level[47].

RESULTS

Weight loss

Although several studies have analyzed the efficacy of SG compared with other techniques, few randomized

Table 1 Randomized trials of bariatric surgery studies including laparoscopic sleeve gastrectomy

Ref.	Country	Follow-up (mo)	Intervention groups	Preoperative BMI (kg/m^2)	Weight loss	T2DM	T2DM remision	T2DM remision criteria
Langer *et al*[53]	Austria	6	SG (10)	48.3	61.4%EWL	10%	NR	
			LAGB (10)	46.7	28.7%EWL	30%		
Himpens *et al*[54]	Belgium	36	SG (40)	39.0	66%EWL	NR		
			LAGB (40)	37.0	48%EWL			
Lee *et al*[55]	Taiwan	12	SG (30)	30.3	76.3%EWL	100%	93%	FG < 126 mg/dL and A1c < 6.5%
			RYGB (30)		94.4%EWL		47%	without hypoglycemic therapy
Karamanakos *et al*[56]	Greece	12	SG (16)	45.1	69.7%EWL			
			RYG (16)	46.6	60.5%EWL			
Kehagias *et al*[57]	Greece	36	SG (30)	44.9	68.5%EWL	16.7%	80%	FG < 126 mg/dL without
			RYGB (30)	45.8	62.1%EWL	16.7%	80%	hypoglycemic therapy
Peterli *et al*[58]	Switzerland	12	SG (11)	44.7	65.6%EWL	0%		
			RYGB (12)	46.7	77.0%EWL	0%		
Schauer *et al*[59]	USA	36	SG (50)	36.2	81%EWL	100%	26.5%	A1c < 6.0% without
			RYGB (50)	37.0	88%EWL		42%	hypoglycemic therapy
			Medical therapy (50)	36.8	13%EWL			
Schauer *et al*[60]	USA	12	SG (50)	36.2	21.1%TWL	100%	29%	A1c < 6.0% without
			RYGB (50)	37.0	24.5%TWL		46%	hypoglycemic therapy
			Medical therapy (50)	36.8	4.2%TWL		0%	
Paluszkiewicz *et al*[61]	Poland	12	SG (36)	46.1	67.6%EWL	27.8%	40%	FG < 100 mg/dL and A1c < 6.0%
			RYGB (36)	48.6	64.2%EWL	38.9%	64.3%	without hypoglycemic therapy
Ramón *et al*[28]	Spain	12	SG (8)	43.5	NR	25.0%	100%	NR
			RYGB (7)	44.2		28.6%	100%	
Vix *et al*[62]	USA	12	SG (45)	45.5	82.9%EWL	8.9%	NR	
			RYGB (45)	47.0	80.3%EWL	8.9%		

BMI: Body mass index; T2DM: Type 2 diabetes mellitus; EWL: Excess weight loss; LABG: Laparoscopic adjustable gastric banding; FG: Fassting glucose; SG: Sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; %EWL: Percentage excess weight loss; %TWL: Percentage total weight loss; NR: Not reported; LAGB: Laparoscopic adjustable gastric banding.

clinical trials showing SG superiority in terms of weight loss compared with other restrictive techniques such as laparoscopic adjustable gastric banding (LAGB) and similar to RYGB have been conducted (Table 1)[28,53-62]. One of the main limitations of those clinical studies was the small sample size that may have accounted for the lack of differences with RYGB. In this regard, Li *et al*[63] conducted a meta-analysis including 21 prospective and 12 retrospective studies with a total of 1375 patients, and no differences were found in excess percentage weight loss (%EWL) at 12 mo between SG (67.1%) and RYGB (68.9%). The few long-term observational studies indicate that although patients regain weight after SG, they achieve a "durable" long-term weight loss. A review of 16 long-term studies revealed %EWL to be 62.3%, 53.8%, 43% and 54.8% at 5, 6, 7 and 8 or more years of follow-up, respectively[64]. Similarly, Himpens *et al*[65] reported that patients regained weight over 3 to 6 years, but most subjects had maintained an %EWL > 50% at 6 years. It is unclear whether this weight regain after SG can justify that RYGB and SG cease to be equally effective in the long term in terms of weight loss. On the one hand, Lim *et al*[66] found no difference up to five years, although it is notable that a high number of patients were lost to follow-up. Moreover, a study by our group found that, unlike what occurs during the first 4 years, RYGB was independently associated with greater weight loss than SG[67]. This weight regain after SG may have several potential reasons. One could be gastric tube dilation. In this respect, Weiner *et al*[68] published a weight regain after SG associated with widening or enlargement of the sleeve after surgery with increased capacity of the gastric tube. A further possible explanation may be incomplete resection of the gastric fundus where ghrelin is produced.

Type 2 diabetes mellitus

Assessment of the effects of SG on diabetes mellitus shows SG to be more than a restrictive bariatric surgery procedure. Clinical studies with a 1-2-years follow-up showed that SG produced higher type 2 diabetes mellitus remission rates than those obtained after other restrictive techniques such as LAGB[69]. Furthermore, as with RYGB, this improvement occurred soon after surgery when significant weight loss had not yet been achieved[70]. These findings could be attributed to changes in the gut hormonal mechanisms previously cited, such as increased GLP-1 secretion or decreased ghrelin. Nevertheless, recent studies seem to show that hormonal mechanisms would be crucial in the short term but would outweigh other factors related to weight loss such as hepatic and peripheral insulin sensitivity in the medium to long term[31].

However, it is noteworthy that in most studies

Table 2 Complication and mortality rates of the different bariatric surgery techniques according to the American College of Surgeons - Bariatric Surgery Center Network

	LSG	LAGB	LRYGB
30-d mortality	0.11	0.05	0.14
1-yr mortality	0.21	0.08	0.34
30-d morbidity	5.61	1.44[1]	5.91
30-d readmission	5.40	1.71[1]	6.47
30-d reoperation	2.97	0.92[1]	5.02[1]

[1]Statistically-significant differences compared with LSG. LSG: Laparoscopic sleeve gastrectomy; LAGB: Laparoscopic adjustable gastric banding; LRYGB: Laparoscopic Roux-Y-gastric bypass.

RYGB showed a trend toward greater improvement in type 2 diabetes mellitus, which may suggest there was a lack of power in those studies. This fact appeared to be confirmed in the meta-analysis of Li *et al*[63] that included 32 studies and 6,526 patients and in which the diabetes remission rate was slightly higher with RYGB (HR = 1.49, 95%CI: 1.04-2.12). This suggests that SG would be placed slightly below RYGB and clearly above other restrictive techniques in terms of type 2 diabetes remission.

Results on the efficacy of SG in long-term diabetes remission are also scant. Abbatini *et al*[23] reported a type 2 diabetes remission rate up to three years of 80.9% with SG, similar to that obtained with RYGB (81.2%) and higher than with LAGB (60.8%). Jiménez *et al*[71] meanwhile detected no differences between SG and RYGB in their cohort of 155 diabetic patients followed for 35.4 ± 13.5 mo.

Hypertension

SG effectiveness in hypertension is greater than other restrictive techniques and below RYGB. In a systematic review, Braghetto *et al*[72] reported a hypertension remission rate of 69% (55%-82%) for SG, 45% (27%-56%) for LAGB and 81% (68%-88%) for RYGB. Similarly, the meta-analysis of Li *et al*[63] detected HR of 1.47 (1.115-1.86) for hypertension remission with RYGB. The superiority of SG over LAGB can be justified by the fact that weight loss is a crucial factor in achieving hypertension remission[73]. Moreover, the superiority of RYGB over SG can be explained by the effects of incretin hormones on blood pressure.

Dyslipidemia

Regarding lipid profile, like other restrictive techniques, SG has a neutral effect on LDL cholesterol concentration[74,75]. Consistent with these results, the hypercholesterolemia remission rate of in the meta-analysis of Li *et al*[63] was higher for RYGB and more clearly so than in other comorbidities (HR = 2.41, 95%CI: 1.87-3.11). Several data support the hypothesis that the main factor involved in lowering LDL cholesterol is the malabsorptive effect of the surgical technique. First, Pihlajamäki *et al*[76] found, as expected based on observed weight loss, decreased serum levels of cholesterol synthesis markers after RYGB or gastric banding. However, a reduction in cholesterol absorption markers was only observed after RYGB, an effect not reported following gastric banding. Second, a relationship exists between the extent of intestinal bypass, which in turn relates to a reduced intestinal absorption area, and the effects on LDL cholesterol. This fact could explain the greater reduction (50%) in LDL cholesterol concentrations seen after purely malabsorptive techniques such as BPD[77] compared to the 17%-20% reported for RYGB[78], a technique with a lower degree of malabsorption.

For HDL cholesterol, SG, like RYBG, produces an increase in its concentration in the short term. We must emphasize that, in a study by our group, the increase in HDL cholesterol was higher for SG[75]. This finding needs to be corroborated by other studies.

Finally, with respect to triglycerides, weight loss is the major factor involved in the reduction in their concentration after different bariatric surgery techniques. As in weight loss, no differences between RYGB and SG in terms of triglyceridemia improvement have been detected[75].

Gastroesophageal reflux

SG may worsen gastroesophageal reflux (GER) owing to increased intragastric pressure, reduced gastric emptying and decreased lower esophageal sphincter pressure. On the other hand, acceleration of gastric emptying and weight loss may improve GER. The results of clinical studies are controversial[65,79]. This controversy could be attributed to methodologic differences in the evaluation of GER and the different follow-up. Some authors proposed that randomized clinical trials should be conducted and that standardized criteria to define GER, validated questionnaires and objective measurements such as pH monitoring should be used to assess the effects of SG[80].

COMPLICATIONS

The introduction of different technical advances has caused a dramatic reduction in bariatric surgery-related mortality. Thus, mortality in RYGB is 10 times higher when performed in open surgery compared with laparoscopy[81]. Mortality after bariatric surgery is currently low and no significant differences exist among the different bariaric surgery techniques according to data from the American College of Surgeons - Bariatric Surgery Center Network including 28616 patients in 25 hospitals in the USA (Table 2)[82]. By contrast, both early complications (< 30 d) and time of surgery for SG yield better results than RYGB and slightly worse than LAGB.

Technical differences among surgeries may cause certain complications that are characteristic of each

technique. It should be noted that up to 20% of subjects who undergo LAGB may require reoperation due to complications related to the gastric band[83]. These reinterventions often occur in the medium to long term and detract from the low rate of early complications after LAGB. Moreover, after SG patients are free of the severe complications of RYGB such as severe hypoglycemia[84], and others such as micronutrient deficiencies or internal hernias are less frequent[85]. Nevertheless, nutritional deficiencies are not uncommon after SG, with multivitamin therapy and postoperative follow-up being recommended[85].

One of the most common and characteristic complications of SG is staple line leak. Although its prevalence is variable, a meta-analysis of 36 studies and 2570 patients showed a frequency of 2.7%[86], but can be < 1% in expert hands[87]. Leaks occur in approximately 90% of cases in the angle of His, leading to detection and therapy being more complex than in RYGB. Different approaches to their management have been proposed, ranging from conservative treatment with fasting until reoperation to a stent or endoscopic treatment by placing clips, fibrin and pyloric dilation to reduce intragastric pressure[88]. Moreover, different staple line reinforcement options have been tested and have proven ineffective to prevent leaks[89].

SPECIAL POPULATIONS

In 1991, the National Institutes of Health (NIH) limited bariatric surgery indication to subjects aged an age between 18 and 60 years and who were very large (BMI > 40 kg/m^2) or large (BMI > 35 kg/m^2) with obesity-related complications[90]. Since then, numerous studies on adolescents, the elderly and subjects with BMI < 35 kg/m^2 have been reported.

As in the general population, the prevalence of obesity in children and adolescents has gradually increased in recent years. In Spain, a rise from 13.9% in 2005 to 19.1% in 2011 was estimated for this specific population[91,92]. There is currently a paucity of data on the long-term efficacy of bariatric surgery in this age range. Data available to date show that SG is safe and effective in the short term and is associated with minimal morbidity and 70% comorbidity resolution[93]. Moreover, SG may have several advantages that render it the technique of choice in obese adolescent candidates for bariatric surgery. On the one hand, SG has a lower risk of late complications such as dumping syndrome or nutritional deficits that patients would suffer for the rest of their lives. Moreover, in cases of significant weight gain, patients could be reoperated on in a second step with a malabsorptive technique.

In patients < 60 years of age RYGB is considered the technique of choice ahead of LAGB given its better risk-benefit ratio[94]. In contrast, in subjects > 60 years, the risk of surgical complications post-RYGB increases significantly and has led some authors to propose LAGB as the technique of choice[95]. No data on the efficacy and safety of SG in patients > 60 years of age are available; however, if the results of patients < 60 years are reproduced, then SG could become the technique of choice in this age range.

In Spain, 17.5% of the population have obesity grade 1 and are therefore without indication for bariatric surgery according to the NIH[96]. Conventional treatment for obesity has proved ineffective in this obese category, which has led to increased research on the effects of bariatric surgery in this weight range. Data currently available are scant and refer only to the short term. Two clinical trials in subjects with BMI < 35 kg/m^2, including SG in one group, have been reported. In the randomized controlled trial by Schauer *et al*[59], 34% of subjects had a BMI < 35 kg/m^2; weight loss and diabetes remission with SG were greater than with conventional treatment and comparable to RYGB (Table 1). Moreover, Lee *et al*[55] randomized 60 subjects to SG or minigastric bypass. In that study, no differences in weight loss between SG and minigastric bypass (94% *vs* 76% EWL, respectively) were detected; however, the diabetes remission rate was higher with minigastic bypass (93% *vs* 47%). We must emphasize that SG was a safe technique in both studies.

Recently, the International Federation for the Surgery of Obesity[97] recommended that bariatric surgery should be considered when sufficient weight loss is not achieved after a reasonable period of time with conventional treatment. The indication of bariatric surgery must be based on more on comorbidities than BMI levels, and these comorbidities should be evaluated in the expected response to the bariatric surgery compared with medical treatment. This statement does not specify what the procedure of choice would be at this BMI level. Given that one of the main reasons to indicate bariatric surgery at this BMI range may be the presence of type 2 diabetes mellitus, SG can play a major role if the same results are reproduced in terms of weight loss and diabetes remission in subjects with BMI > 35 kg/m^2.

Patients with extreme obesity have a higher risk of perioperative complications and mortality than those with a BMI < 50 kg/m^2[98]. As mentioned previously, SG was initially designed as a first step before a BPD in obese subjects at high risk[1,2]. SG as a single technique does not seem appropriate for extremely obese patients since a high percentage maintain a BMI > 40 kg/m^2 in the medium term[99]. Weight loss and improvement in comorbidities after SG are associated with improved Anesthesiologist American Society (ASA) risk and consequently a reduced risk of surgical complications[100]. This approach of two steps being safer that one step has proved effective in terms of weight loss and improvement in comorbidities in extremely-obese patients[86].

Table 3 Sleeve gastrectomy may be preferable to other procedures in the following situations

Extreme obesity (BMI > 50 kg/m^2): first-step procedure
ASA IV morbidly-obese patient
Absence of hypercholesterolemia
To avoid drug malabsorption
Extreme ages
BMI of 35-40 kg/m^2 with comorbidity
Class I obesity
Crohn's disease
Prevent potential consequences of hypoglycemia in specific occupations

BMI: Body mass index.

FUTURE PERSPECTIVES AND CONCLUSION

It can be concluded that SG can indeed be considered more than a restrictive bariatric surgery procedure. Its benefits are far more than those associated with a reduction in gastric volume and its results in terms of weight loss and improvement of comorbidities are superior to those obtained with other restrictive procedures. Additionally, SG offers further advantages such as high efficiency, low technical complexity and low rate of surgical complications. All these characteristics render SG preferable to other procedures in certain situations (Table 3) and may, in a near future, place it as the next gold standard in bariatric surgery at the expense of RYGB (Table 4). However, long-term studies aimed at establishing SG as non-inferior relative to the current gold standard, RYGB, are required.

Table 4 Clinical outcomes of sleeve gastrectomy and Roux-en-Y gastric bypass

	SG	RYGB
Weight loss	No differences with RYGB 67.1 %EWL at 12 mo[63]	No differences with SG 68.9% EWL at 12 mo[63]
Type 2 diabetes mellitus remission	Early improvement before significant weight loss More effective than other restrictive techniques	Slightly more effective than SG. HR 1.49, 95%CI: 1.04-2.12 for type 2 diabetes mellitus remission in favor of RYGB[63]
Hypertension remission	Greater efficacy than other restrictive techniques 69% (55-82) Hypertension remission for SG and 45% (27-56) for LAGB[72]	More effective than SG HR of 1.47, 95%CI: 1.115-1.86 for Hypertension remission in favor of RYGB[63]
Dyslipidemia remission	Same as other malabsorptive techniques, no hypercholesterolemia improvement	Clearly more effective than SG. HR = 2.41, 95%CI: 1.87-3.11 for Dyslipidemia remission in favor of RYGB[63]
Mortality	No differences (detailed in table 2)	No differences
Surgical complications	Less surgical time, lowest 30-d morbidity, 30-d readmission and 30-d reoperation. (detailed in table 2) Characteristic complications: staple line leaks (2.7%[86]; < 1% in expert hands[87])	Increased risk of nutritional deficiencies Characteristic complications: severe hypoglycemia
Long-term results	Limited evidence	Effective and safe in the long term
Other advantages	Possibility of conversion to a malabsorptive surgery	

SG: Sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; %EWL: Percentage excess weight loss; HR: Hazard ratio; LABG: Laparoscopic adjustable gastric banding.

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REVIEW

Inflammation: A novel target of current therapies for hepatic encephalopathy in liver cirrhosis

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Abstract

Hepatic encephalopathy (HE) is a severe neuropsychiatric syndrome that most commonly occurs in decompensated liver cirrhosis and incorporates a spectrum of manifestations that ranges from mild cognitive impairment to coma. Although the etiology of HE is not completely understood, it is believed that multiple underlying mechanisms are involved in the pathogenesis of HE, and one of the main factors is thought to be ammonia; however, the ammonia hypothesis in the pathogenesis of HE is incomplete. Recently, it has been increasingly demonstrated that inflammation, including systemic inflammation, neuroinflammation and endotoxemia, acts in concert with ammonia in the pathogenesis of HE in cirrhotic patients. Meanwhile, a good number of studies have found that current therapies for HE, such as lactulose, rifaximin, probiotics and the molecular adsorbent recirculating system, could inhibit different types of inflammation, thereby improving the neuropsychiatric manifestations and preventing the progression of HE in cirrhotic patients. The anti-inflammatory effects of these current therapies provide a novel therapeutic approach for cirrhotic patients with HE. The purpose of this review is to describe the inflammatory mechanisms behind the etiology of HE in cirrhosis and discuss the current therapies that target the inflammatory pathogenesis of HE.

Key words: Inflammation; Hepatic encephalopathy; Pathogenesis; Treatment

Core tip: Currently, inflammation appears to play a critical role in the pathogenesis of hepatic encephalopathy (HE) and is gradually being considered a critical therapeutic target for HE in patients with liver cirrhosis. Current therapies for HE, including lactulose, rifaximin, probiotics and the molecular adsorbent recirculating system, have been found to improve clinical manifestations and prevent the progression of HE by ameliorating inflammation in cirrhotic patients. This review will provide an overview of the inflammatory pathogenesis of HE, focusing on the recent findings on its therapeutic manipulation.

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INTRODUCTION

Hepatic encephalopathy (HE) is a morbid neuropsychiatric complication resulting from decompensated liver disease or portosystemic shunting and is characterized by disturbances of both cognitive and motor functions, ranging from subtle psychometric abnormalities to coma[1]. According to the updated guideline, HE is classified into three different types: A, B and C (Table 1)[1]. Subsequently, based on the severity of the clinical manifestations, type C HE is subdivided into covert HE (including minimal HE and West-Haven grade Ⅰ HE) and overt HE (West-Haven grades Ⅱ-Ⅳ HE)[1]. Cirrhotic patients with overt HE present a series of severe neuropsychiatric manifestations, such as asterixis, dyspraxia, and even progressing to stupor and coma. In contrast, minimal HE exhibits trivial cognitive deficits that are only detected using psychometric or neurophysiological tests without the obvious manifestations of overt HE[2]. Minimal HE impairs cognitive functions and health-related quality of life in cirrhotic patients and is considered an important predictive factor for the development of overt HE[3,4].

In spite of several decades of investigation, the exact mechanisms responsible for the pathogenesis of HE still have not been fully elucidated. Ammonia is universally regarded as the major precipitating factor in the pathogenesis of HE, and the vast majority of the current therapies for HE are centered on reducing the production and absorption of ammonia; however, several studies have suggested that the concentration of ammonia can be elevated in the absence of symptoms of HE, and the ammonia concentration is not always consistent with the severity of HE in cirrhotic patients[5-7]. Furthermore, approximately 20% of patients with chronic liver failure and HE have been found to be non-responsive to lactulose treatment, and it has been demonstrated that non-absorbable disaccharides do not reduce the mortality of cirrhotic patients with HE[8,9]. Apart from hyperammonemia, various other pathogenic mechanisms, such as the γ-aminobutyric acid (GABA) theory, the benzodiazepine theory, the manganese theory and the theory of oxidative/nitrosative stress, have been implicated in the development of HE[1]. Currently, it is believed that HE is a result of multiple pathophysiologic mechanisms that induce the functional impairment of the central nervous system.

Over the past decade, increasing evidence has indicated that inflammation, including systemic inflammation, neuroinflammation and endotoxemia, plays an important role in the pathogenesis of HE, and inflammation is gradually being considered a critical therapeutic target for HE in cirrhotic patients[5,10,11]. Meanwhile, the current therapies for HE, such as lactulose, rifaximin, probiotics and the molecular adsorbent recirculating system (MARS), have been found to modulate the inflammatory response and lower pro-inflammatory mediator levels, which help to improve the clinical manifestations and delay the progression of HE in cirrhotic patients (Figure 1)[12-15]. These recent findings have demonstrated the possibility of these therapies in ameliorating inflammation and providing a novel and promising therapeutic alternative for patients with HE secondary to liver cirrhosis. This review summarizes the inflammatory mechanisms implicated in the pathogenesis of HE and evaluates the evidence of current therapies that target the inflammatory pathogenesis of HE in clinical practice.

Table 1 Classification of hepatic encephalopathy[1]

Type	Definition	Subdivision
A	Caused by acute liver failure	
B	Secondary to portosystemic bypass or shunting without intrinsic liver disease	
C	Results from chronic liver disease, especially decompensated cirrhosis	Covert HE (minimal HE and West-Haven grade Ⅰ HE)
		Overt HE (West-Haven grades Ⅱ-Ⅳ HE)

HE: Hepatic encephalopathy.

INFLAMMATORY PATHOGENESIS OF HE IN CIRRHOSIS

Systemic inflammation

Cirrhotic patients are commonly found to have substantial disturbances of intestinal flora, with significant small intestinal overgrowth of potentially pathogenic Gram-negative bacteria, including *Enterobacteriaceae*, *Alcaligenaceae* and *Streptococcaceae*[16]. Furthermore, intestinal vascular congestion caused by portal hypertension, oxidative damage of the intestinal mucosa and the absence of mucosal immunoglobulin A secretion in cirrhosis have been demonstrated to result in increased intestinal permeability and intestinal barrier dysfunction[17]. Furthermore, ammonia induces neutrophil and macrophage dysfunction and impairs phagocytosis, which may culminate in a "sepsis-like" immune paralysis[18,19]. These mechanisms synergistically induce the bacterial translocation that includes the migration of bacteria or bacterial byproducts from the small intestine to the systemic circulation, ultimately leading to spontaneous bacterial peritonitis and further systemic infection in patients with liver cirrhosis[20]. The study by Caly *et al*[21] has indicated

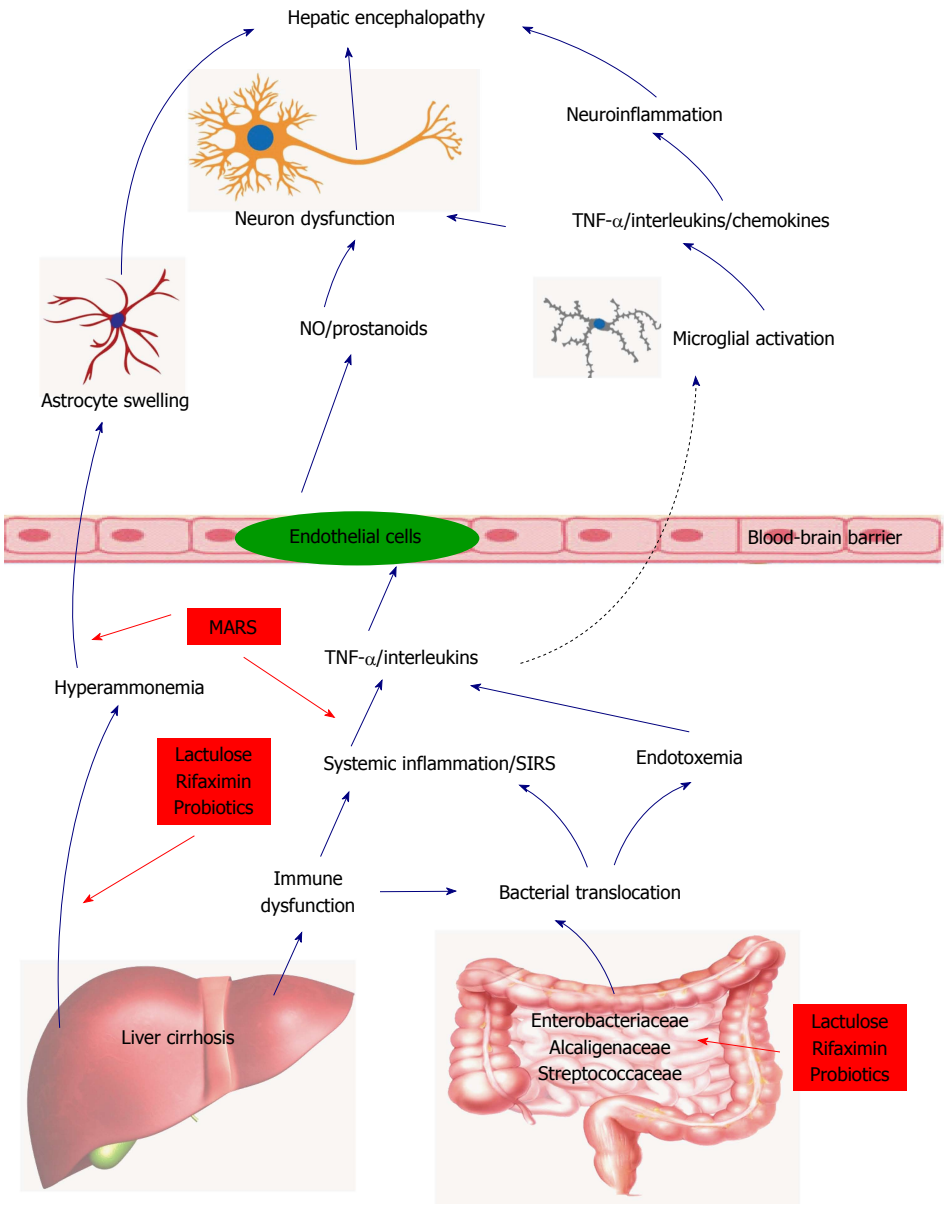

Figure 1 The inflammatory pathogenesis of hepatic encephalopathy in liver cirrhosis. Lactulose, rifaximin, and probiotics not only reduce the circulating levels of ammonia but also modulate intestinal flora, lower the endotoxemia and inhibit the production of pro-inflammatory cytokines. MARS treatment also decreases the circulating levels of ammonia and pro-inflammatory cytokines.

that infection is the reason for hospital admission in 30% to 50% of cirrhotic patients, and 15% to 35% of them ultimately develop nosocomial infections during their hospital stay. Predominant infections presented in decompensated cirrhosis are spontaneous bacterial peritonitis, urinary tract infections, nosocomial pneumonia, sepsis and even systemic inflammatory response syndrome (SIRS)[22].

Circulating levels of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukins (ILs), are significantly elevated in decompensated cirrhotic patients[23]. These cytokines cannot exert their direct effects on the brain because they are unable to directly cross the blood-brain barrier (BBB). However, recent studies have demonstrated that TNF-α and IL-1β can influence the permeability of the BBB, and these peripheral cytokines exert their effects on the brain *via* the following three pathways: (1) peripheral tissues convey signals to the brain through the activation of the vagus nerve's afferent neurons; (2) the brain vasculature sends signals through secondary messengers that are produced in response to cytokines, such as nitric oxide (NO) and prostanoids; and (3) cytokines enter brain areas that lack the BBB, and, subsequently, act at the brain parenchyma[24].

There is mounting clinical evidence for the role of systemic inflammation in the development of overt and minimal HE in cirrhotic patients. Serum concentrations of TNF-α and IL-6 have been found to correlate positively with the severity of overt HE in decompensated cirrhotic patients, and TNF-α is believed to be strongly involved in the pathogenesis of HE due

to chronic liver failure[25-27]. Furthermore, systemic infection/SIRS, but not ammonia, was correlated with increasing grades of overt HE in cirrhotic patients with grades Ⅲ-Ⅳ HE[5]. Similarly, serum levels of TNF-α, IL-6 and IL-18 were associated with the severity of minimal HE, and serum levels of IL-6 and IL-18 might have the capacity to identify cirrhotic patients with and without minimal HE[28,29]. In addition, Shawcross *et al*[6] have reported that the presence and severity of minimal HE were not correlated with ammonia concentrations, but serum levels of inflammatory markers, including C-reactive protein and IL-6, were significantly higher in cirrhotic patients with minimal HE compared with those without, which indicated that systemic inflammation is a critical determinant of the presence and severity of minimal HE.

Neuroinflammation

Neuroinflammation is considered to be an inflammatory response in the brain and is featured by microglial activation and the cerebral production of pro-inflammatory mediators[10]. Neuroinflammation is closely associated with systemic inflammation. Vascular endothelial cells, along with astrocytes, are a major constituent of the BBB. Endothelial cells induce the release of different pro-inflammatory mediators into the brain when they are stimulated by systemic inflammation[30]. For instance, endothelial cells have receptors for TNF-α and IL-1β, and these receptors convey signals that induce the synthesis of secondary messengers in the brain, such as NO and prostanoids[31]. Moreover, microglial cells constitute the resident macrophages of the brain and can be activated by pro-inflammatory mediators, releasing various types of chemokines with inflammatory properties[32]. These mechanisms have been demonstrated to contribute to the development of neuroinflammation in the brain.

Evidence for the role of neuroinflammation in the pathogenesis of HE due to cirrhosis has recently been provided by several animal experiments. Motor deficits, psychomotor slowing and hypokinesia are commonly presented in cirrhotic patients with HE, which can be simulated in rats with a portacaval shunt (PCS) and bile duct ligation (BDL), according to the recommendation by the International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN)[33]. A study by Cauli *et al*[34] revealed that PCS rats exhibited increased levels of IL-6 and increased activities of cyclooxygenase and inducible NO synthase in the cerebral cortex, indicating the presence of neuroinflammation. Subsequently, chronic treatment with an anti-inflammatory drug, ibuprofen, could normalize the activities of cyclooxygenase and inducible NO synthase and completely restore the learning ability of PCS rats. In addition, BDL activated the microglia in the cerebellum, increased levels of inducible NO synthase, IL-1β and prostaglandin E2, and impaired the rats' cognitive and motor functions. Similarly, ibuprofen also ameliorated neuroinflammation and restored the cognitive and motor functions of BDL rats[35]. These findings indicate that neuroinflammation contributes to cognitive and motor alterations in experimental animal models of HE and point to the possibility that the anti-inflammatory treatment may improve cognitive deficits in cirrhotic patients with HE. However, in cirrhotic patients with HE, microglial activation has not been found to be correlated with increased mRNA expression of TNF-α, IL-1β and IL-6 in the cerebral cortex[36]. Furthermore, compared with the controls, mRNA profiles of these cytokines remained unchanged in the brains of cirrhotic patients with HE, despite an up-regulation of genes associated with microglial activation[37]. The underlying reason responsible for this inconsistency is unclear; thus, further studies are required to clarify the role of neuroinflammation in the inflammatory pathogenesis of HE secondary to liver cirrhosis.

Endotoxemia

Due to intestinal bacterial translocation and portosystemic shunting, endotoxin, the lipopolysaccharide in the outer membrane of Gram-negative bacteria, enters the systemic circulation and is responsible for long-standing endotoxemia[17]. Endotoxin is able to activate immune cells either through activating Toll-like receptors or by inducing the production of pro-inflammatory cytokines[38]. Similar to pro-inflammatory cytokines, endotoxin also cannot cross the BBB, but it increases BBB permeability and acts on the brain parenchyma through endothelial cell receptor interactions with the downstream production of NO and prostanoid[39,40].

Henry *et al*[41] found that a peripheral lipopolysaccharide injection induced a hyperactive microglial activation in the brains of mice and resulted in a significant induction of mRNA expression of both IL-1β and IL-10 in the cerebral cortex of aged mice. In addition, lipopolysaccharide that was injected into the rats' hippocampus could lead to microglial activation and increased production of TNF-α and IL-1β in the hippocampus, inducing a reduction of glutamatergic transmission that led to learning and memory deficits without neuronal cell death[42]. Endotoxemia without sepsis has been reported in patients with liver cirrhosis and was found to be associated with an increased incidence of overt HE and mortality in these patients[43]. Moreover, a study by Jain *et al*[11] showed that serum levels of endotoxin were correlated with the severity of minimal HE due to cirrhosis. Endotoxemia may play a crucial role in the inflammatory pathogenesis of HE, especially in compensated cirrhotic patients without evidence of proven infection.

Ammonia-inflammation synergism

Decompensated cirrhosis results in both systemic inflammation and hyperammonemia. At the cellular level, TNF-α and IL-6 influence the permeability of the BBB, and human cerebrovascular endothelial cells

increased ammonia uptake when exposed to TNF-α *in vitro*[44]. On the background of hyperammonemia, neuroinflammation involving both pro-inflammatory and neurotransmitter pathways can be induced by systemic inflammatory stimulus[45]. Furthermore, hyperammonemia leads to lactate accumulation in the brain, and both systemic inflammation and brain lactate accumulation lead to microglial activation and the increased production of TNF-α, IL-1β and IL-6[46]. Therefore, it is believed that ammonia potentially acts in concert with inflammation in a synergistic manner in the pathogenesis of HE.

Marini *et al*[47] found that mice with chronic hyperammonemia exhibited prolonged cognitive and motor deficits when treated with a lipopolysaccharide stimulus. Furthermore, chronic hyperammonemia has been found to induce microglial activation and subsequent neuroinflammation that were associated with the cognitive and motor deficits of BDL rats[35]. In a study by Jover *et al*[48], although both BDL and ammonia-fed BDL rats exhibited microglial activation in the brain, ammonia-fed BDL rats showed severe motor deficits compared to BDL rats, whose motor functions seemed only mildly influenced. Likewise, cirrhotic patients with systemic inflammation and hyperammonemia also showed significant impairments of cognitive and motor functions. For example, Shawcross *et al*[49] have reported that systemic infection/SIRS exacerbated the neuropsychological deterioration induced by hyperammonemia in cirrhotic patients with overt HE. Moreover, in cirrhotic patients with minimal HE, there were considerable cognitive impairments following induced hyperammonemia during infection, but not after its resolution[6]. In addition, Felipo *et al*[50] have found that hyperammonemia or inflammation alone did not result in cognitive impairments, but the synergistic effect of hyperammonemia and inflammation was sufficient to induce cognitive impairments in cirrhotic patients with minimal HE. This evidence suggests that, on the background of cirrhosis, inflammation and its mediators modulate the cerebral effect of hyperammonemia, and there is a synergistic relationship between hyperammonemia and inflammation in the pathogenesis of HE in liver cirrhosis.

CURRENT THERAPIES TARGETING THE INFLAMMATORY PATHOGENESIS OF HE

Non-absorbable disaccharides

Non-absorbable disaccharides, including lactulose and lactitol, lower the production and absorption of ammonia and are traditionally considered the current mainstay therapy for HE. Recently, emerging studies have indicated that non-absorbable disaccharides not only reduce circulating levels of ammonia but also decrease those of pro-inflammatory cytokines and endotoxin. For example, Jia *et al*[51] showed that lactulose lowered the level of hyper-endotoxemia, improved the cognitive and motor functions, and decreased the incidence of minimal HE in a rat model. Similarly, in cirrhotic patients with minimal HE, lactulose regulated the stool microbiome, lowered the level of serum endotoxin, inhibited the production of TNF-α, IL-2, IL-6 and IL-13, and consequently, improved their psychometric function[12]. Moreover, a study by Jain *et al*[11] revealed that lactulose inhibited intestinal bacterial overgrowth, significantly reduced the serum concentrations of TNF-α, IL-6, IL-18 and endotoxin, and subsequently improved cognitive functions in cirrhotic patients with minimal HE. By contrast, lactulose withdrawal resulted in a mixed inflammatory response and cognitive deterioration in cirrhotic patients with minimal HE[52]. In addition, lactulose improved cognitive and motor functions in cirrhotic patients with minimal HE, which helped to improve health-related quality of life, prevent motor vehicle accidents and reduce societal costs[3,53]. For cirrhotic patients with overt HE, a meta-analysis of randomized clinical trials showed that lactulose had beneficial effects on overt HE manifestations, and lactulose has been demonstrated to be efficacious in the secondary prevention of overt HE[54,55]. Given their cost and availability, non-absorbable disaccharides are likely to remain a well-used therapy in HE.

The side effects of non-absorbable disaccharide treatment include flatulence, diarrhea, abdominal pain and intestinal malabsorption, resulting in frequent noncompliance in cirrhotic patients with HE[56]. Additionally, despite substantial evidence of the beneficial effects of non-absorbable disaccharides, their efficacy and safety for HE have been recently questioned. For example, a Cochrane review revealed that clinical trials with high methodological quality found no significant effect of non-absorbable disaccharides on the risk of no improvement in HE and mortality[56]. Thus, there has been insufficient evidence to determine whether non-absorbable disaccharides are of benefit to cirrhotic patients with HE, and more high-quality studies are required in the future.

Rifaximin

Antibiotics are able to eliminate pathogenic Gram-negative bacteria in the intestinal tract, inhibit bacterial translocation, and consequently, decrease the overproduction of pro-inflammatory cytokines and endotoxin. Prominent antibiotics used in the treatment of HE are neomycin, metronidazole, vancomycin and rifaximin; however, extensive side-effect profiles and the potential for bacterial antimicrobial resistance have limited the utility of most of these antibiotics in treating HE, with the exception of rifaximin, which is the only systematically studied antibiotic and has substantial clinical evidence[57].

Rifaximin is a non-absorbable antibiotic with wide antimicrobial activity against both aerobic and anaerobic Gram-negative bacteria[58]. Recently,

Vlachogiannakos *et al*[59] found that selective intestinal decontamination with a rifaximin regimen significantly ameliorated endotoxemia in patients with decompensated alcohol-related cirrhosis. Furthermore, the studies by Kalambokis *et al*[60,61] revealed that rifaximin could decrease serum concentrations of TNF-α, IL-6 and endotoxin in patients with alcoholic cirrhosis. In cirrhotic patients with minimal HE, rifaximin was found to alter intestinal bacterial linkages with metabolites without considerable changes in the intestinal flora, decrease the circulating levels of endotoxemia, and improve cognitive function[13]. These results indicate that rifaximin can regulate the intestinal flora, reduce the production of endotoxin and pro-inflammatory cytokines and ultimately improve cognitive function in cirrhotic patients with HE.

The clinical efficacy of rifaximin as a treatment for HE has been extensively explored in several clinical trials. For example, a prospective randomized, double-blind, controlled trial found that rifaximin significantly improved mental state, electroencephalogram irregularities and portal-systemic encephalopathy index in cirrhotic patients with grades Ⅰ-Ⅲ acute HE[62]. Compared with lactulose, treatment of HE with rifaximin was correlated with decreased hospitalization duration, lower hospitalization expenses and better clinical manifestations[63]. Moreover, a large, double-blinded, randomized, controlled study by Bass *et al*[64] has demonstrated that rifaximin not only significantly reduced the risk of hospitalization involving HE but also effectively maintained remission from HE. In these clinical trials, rifaximin was well-tolerated, and fewer adverse events were reported compared with treatments with non-absorbable disaccharides. These results suggest that rifaximin may be an effective alternative treatment to non-absorbable disaccharides in treating HE, with an acceptable side effect profile.

As mentioned above, lactulose has no beneficial impact on the mortality of cirrhotic patients with HE. Different from lactulose, Sharma *et al*[65] have demonstrated that rifaximin significantly reduced the mortality of cirrhotic patients with HE, and the combination of lactulose plus rifaximin was more effective than lactulose alone in the treatment of HE. Furthermore, a systematic review with meta-analysis by Kimer *et al*[66] showed that rifaximin increased the proportion of patients whose neuropsychiatric manifestations improved and reduced mortality, which indicated that rifaximin should be used for secondary prevention of HE; however, although rifaximin did result in lower readmission rates for HE at half a year, the addition of rifaximin to lactulose for treating acute HE did not reduce hospital length of stay[67]. Therefore, the efficiency of combined treatment with lactulose plus rifaximin should be further evaluated by more randomized and controlled clinical trials.

Probiotics

Probiotics are living beneficial bacteria in the intestinal tract, and they are able to inhibit the activity of bacterial ureases, modulate intestinal pH values, and ultimately, reduce ammonia absorption[68]. Recently, probiotics have been reported to inhibit the bacterial activators of Toll-like receptors (TLRs), lower endogenous levels of IL-10 and TLR-4 expression, and ultimately, restore neutrophil phagocytic activity in alcohol-related cirrhotic patients[69]. Furthermore, probiotics modulate derangements in gut microbiota *via* inhibiting the overgrowth of pathogenic bacteria and prevent bacterial translocation, thus significantly lowering serum levels of endotoxin, which may help to inhibit the production and activity of pro-inflammatory cytokines[70].

The past decade has witnessed an upsurge of interest in the utility of probiotics for treating minimal HE in cirrhotic patients. For instance, in a phase Ⅰ clinical trial, the probiotic *Lactobacillus GG* reduced *Enterobacteriaceae*, increased the relative abundance of *Clostridiales Incertae* Sedis XIV and *Lachnospiraceae*, and further decreased endotoxemia and serum concentrations of TNF-α in cirrhotic patients with minimal HE, suggesting that *Lactobacillus GG* modulates intestinal dysbiosis and prevents systemic inflammatory response in these patients[14]. During this study, however, there was no significant improvement in cognitive functions before or after *Lactobacillus* GG treatment, and *Lactobacillus* GG was correlated with a markedly higher percent of self-limited diarrhea, which gives impetus to further studies regarding probiotics for treating minimal HE in cirrhotic patients. In addition, synbiotics (*i.e*., probiotics and fermentable fiber) treatment significantly increased the fecal content of the non-urease-producing *Lactobacillus* species at the expense of these other bacterial species, which was correlated with a marked decrease in endotoxemia and a reversal of minimal HE in 50% of cirrhotic patients[71]. Furthermore, a multi-strain probiotic compound containing *Lactobacillus*, *Bifidobacterium* strains and *S. thermophiles* improved the neuropsychological manifestations of cirrhotic patients with minimal HE, and these probiotics had longer-term therapeutic effects than lactulose[72]. Moreover, probiotic VSL#3 was found to be non-inferior to the standard therapy, lactulose, in treating minimal HE[73]. Nevertheless, a meta-analysis by Shukla *et al*[74] revealed that lactulose appeared to have the most beneficial effect in minimal HE, followed by probiotics and synbiotics. Probiotics may take the place of lactulose for the standard treatment of minimal HE, but this possibility should be evaluated by more controlled trials that compare their efficacy.

In the above-mentioned clinical trials, the side effects of probiotics were reported to be mild, and there have been no reported adverse events related to treatment with probiotics in minimal HE; however, a randomized, controlled trial by Besselink *et al*[75] revealed that probiotics did not reduce the risk of infectious complications, and they were correlated

with an increased risk of mortality in patients with predicted severe acute pancreatitis. Similarly, whether oral supplementation with probiotics may induce infectious complications in cirrhotic patients with HE requires further investigation, especially in those with severe infection. Furthermore, an updated meta-analysis by Xu *et al*[76] found that although probiotics significantly prevented the development of HE, they did not affect serum ammonia levels or cirrhotic patients' mortality. In addition, a Cochrane review by McGee *et al*[77] showed that there was no sufficient evidence of clinically significant improvement in HE treated with probiotics, and probiotics were especially shown to have no benefit to mortality. Therefore, the use of probiotics for cirrhotic patients with HE cannot be currently recommended, and rigorous clinical evaluation in randomized controlled trials is required.

MARS

Extracorporeal albumin dialysis, especially MARS, is a new method of hemodiafiltration in which blood is dialyzed against an albumin-containing solution across a high-flux membrane, which allows for the combined elimination of albumin-bound and water-soluble toxins. A Cochrane review by Liu *et al*[78] revealed that MARS treatment reduced the mortality of patients with acute-on-chronic liver failure (ACLF) and had a beneficial effect on HE. For example, Dominik *et al*[79] reported that treatment with MARS decreased the serum concentrations of TNF-α and IL-6 in patients with ACLF due to cirrhosis. In a study by Guo *et al*[15], MARS treatment significantly decreased serum levels of TNF-α, IL-6, IL-8 and INF-γ, which was associated with improvements of HE in ACLF. Furthermore, results of a study by Sen *et al*[80] showed that MARS treatment improved HE manifestations in patients with inflammation-related ACLF, and the main therapeutic effect of MARS was on other inflammatory mediators, such as NO, that were reduced by a combination of their elimination and decreased production. Taken together, these results suggest that MARS is regarded as a potentially effective alternative for eliminating inflammatory mediators from the circulation and ameliorating HE manifestations in ACLF patients who fail to respond to conventional therapy.

MARS has been demonstrated to be beneficial for HE secondary to ACLF; however, its efficacy did not appear to be associated with alterations in the serum levels of pro-inflammatory cytokines. In patients with ACLF, Stadlbauer *et al*[81] found that cytokines were eliminated from plasma by MARS treatment; however, MARS could not lower the serum cytokines levels. This difference may be attributed to increased cytokine production in ACLF. Furthermore, a prospective, multi-center trial by Hassanein *et al*[82] revealed that although MARS treatment improved grades Ⅲ and Ⅳ HE earlier than standard medical therapy in ACLF patients, there was no significant difference in SIRS scores between MARS treatment and standard medical therapy. Moreover, although the neurological manifestations of patients with HE were improved, no significant change in the serum concentrations of TNF-α, IL-6 and IL-8 was observed before *vs* after MARS treatment in ACLF, and MARS treatment did not exhibit a clearly identifiable efficacy in eliminating these circulating cytokines[83]. Although the above-mentioned clinical trials support the fact that MARS treatment is of benefit to the improvement of HE, these trials did not specifically evaluate the efficacy of MARS treatment in HE and were designed to only examine the improvements of ACLF. Therefore, the therapeutic effect of MARS treatment on the inflammatory pathogenesis of HE awaits the completion of further clinical trials.

CONCLUSION

HE is a serious neuropsychiatric complication of liver cirrhosis, and inflammation is a critical participating factor in the pathogenesis of HE. As mentioned above, existing therapies including lactulose, rifaximin, probiotics and MARS have been demonstrated to be beneficial for HE in cirrhotic patients by ameliorating the inflammatory pathogenesis of HE. These recent findings indicate that inflammation should be considered an important therapeutic target for HE and also point to the possibility that anti-inflammatory therapies will be promising alternatives for the treatment of HE in cirrhotic patients; however, the efficacy of these alternatives has not been fully confirmed and their safety is still questioned. Furthermore, the influence of these alternatives on the prognosis of cirrhotic patients with HE has remained controversial. Therefore, in the future, more multi-center, randomized, controlled trials are required to evaluate the efficacy and safety of these alternatives.

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MINIREVIEWS

Role of oats in celiac disease

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Abstract

A gluten-free diet is currently the only effective means of treating individuals with celiac disease. Such a diet enables celiac patients to control their symptoms and avoid various complications associated with this condition. However, while the quality of gluten-free foods has significantly improved during recent decades, maintenance of a gluten-free diet does not necessarily ensure adequate nutritional intake. Because oats are an important source of proteins, lipids, vitamins, minerals, and fibre, their inclusion in a gluten-free diet might improve the nutritional status of a celiac patient. Although oats are included in the list of gluten-free ingredients specified in European regulations, their safety when consumed by celiac patients remains debatable. Some studies claim that pure oats are safe for most celiac people, and contamination with other cereal sources is the main problem facing people with this disease. However, it is necessary to consider that oats include many varieties, containing various amino acid sequences and showing different immunoreactivities associated with toxic prolamins. As a result, several studies have shown that the immunogenicity of oats varies depending on the cultivar consumed. Thus, it is essential to thoroughly study the variety of oats used in a food ingredient before including it in a gluten-free diet.

Key words: Oats; Celiac disease; Gluten-free diet

Core tip: Symptoms of celiac disease are triggered by an abnormal reaction to gluten, and the only treatment for celiac disease is the patient's adherence to a strict gluten-free diet. While inclusion of oats in a gluten-free diet might improve its overall nutritional value, their use in such diets remains controversial. This review summarizes recent advances made in understanding the nutritional properties of oats and their role in celiac disease.

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INTRODUCTION

Celiac disease (CD) is a lifelong autoimmune disease characterized by an aberrant inflammatory response to dietary gluten in genetically susceptible individuals. CD is one of the most common chronic digestive disorders, and afflicts about 1% of the population in Western countries; moreover, recent studies suggest that its prevalence is increasing[1,2]. Certain individuals show a strong and complex genetic predisposition to this disease. Although 95% of celiac patients are *HLA-DQ2* or *-DQ8* positive, the presence of these alleles is not strongly predictive for the disease[3,4]. In recent years, the clinical spectrum of CD patients has been expanded to include asymptomatic individuals, as well as individuals with minimal symptoms (the most difficult to detect) and extra-intestinal symptoms[5-7]. However, regardless of its symptomatic presentation, virtually all cases of active CD occur after the susceptible individual has received dietary exposure to the environmental antigen, gluten. In patients with CD, the ingestion of gluten proteins contained in wheat, barley, and rye results in characteristic inflammation, villous atrophy, and crypt hyperplasia in the upper small intestine[5].

During the early 1980s, the spectrum of gluten-related disorders was relatively simple: CD and dermatitis herpetiformis (CD of the skin). While a gluten-free diet (GFD) is still recommended for all patients with CD, the area of CD research is changing rapidly, and wheat allergies, gluten ataxia, and nonceliac gluten sensitivity have recently been added as new gluten-related topics for study[8]. The only treatment for these disorders remains adherence to a GFD; however, many patients experience persistent CD-related symptoms despite their best efforts to avoid dietary gluten. A GFD is expensive and difficult to maintain because many products made from gluten-containing grains are Western dietary staples. Moreover, the contents of a GFD do not always ensure that an individual receives adequate nutrition[9,10]. In fact, medical problems related to inadequate nutrition have been described in CD patients following their long-term treatment with a strict GFD. Such observations might possibly be explained by the composition and nutritional quality of commercially available gluten-free products. While individuals on a GFD need to replace wheat, barley, rye, and their derivatives with foods derived from naturally gluten-free cereal grains (*e.g.*, rice, corn, buckwheat, sorghum, *etc.*), but the recommended amounts of fibre, iron and calcium can be more difficult to obtain on such a diet and good planning is required[11]. Oats contain both soluble and insoluble dietary fibre, B-complex vitamins, iron, and proteins[12,13], and have recently been considered for inclusion in a GFD. However, while oats might improve the nutritional value of a GFD, their safety for consumption by celiac patients has been the subject of controversy.

NUTRITIONAL AND PHARMACOLOGICAL PROPERTIES OF OATS

Composition

Oat grain is characterized by its good taste and dietetic properties, as well as an ability to stimulate metabolic changes in the bodies of humans and animals. Furthermore, oat grain is a rich source of proteins with favourable amino acid contents and high nutritional value, and as other beneficial ingredients including dietary fibre, antioxidants, vitamins, phenolic compounds, minerals, and essential unsaturated fatty acids[14-16]. When compared with other cereal grains, oat grain contains larger amounts of total protein and crude fat, and a small amount of crude fibre. The major nutritional components of oats are shown in Table 1.

Health benefits

Several studies have described oats as a functional food with the ability to lower blood cholesterol and sugar levels, reduce hypertension, help control childhood asthma, reduce body weight, and also provide immunomodulatory, antioxidant, and antiatherogenic effects[14,17-19] (Table 2). Oats also contain significant amounts of vitamins, minerals, fibre, and phytochemicals that regulate intestinal transit times and increase the production of butyrate and/or other faecal short chain fatty acids produced by gut microflora. As a result, the long-term dietary intake of oats or oat bran might benefit patients suffering from inflammatory bowel disease, ulcerative colitis, colorectal adenoma or cancer. However, further studies are required to accurately assess the benefits provided by increased oat consumption when treating bowel disorders[20].

Additionally, due to the well-established effect of oats on the risk for coronary heart disease, in 1997 the United States Food and Drug Administration (FDA) approved the heart-health benefit claim shown on the label of many foods containing soluble fibre derived from oats. Moreover, in 2010, a European Food Safety Authority (EFSA) panel concluded that current scientific evidence supports the following two-part statement: "Oat β-glucan has been shown to lower/reduce blood cholesterol. Blood cholesterol lowering may reduce the risk of coronary heart disease".

OATS AND CELIAC DISEASE

Oat avenins

As the prolamin components of oat seeds, the avenins are known to exist as both monomers and

Table 1 Main nutritional components of oats

	Components	Properties	Ref.
Proteins	Albumins, globulins, prolamins, and glutenins	Oats are distinct among cereals due to their higher protein concentration and distinct protein composition. The major storage proteins are globulins	[50, 51]
Carbohydrates	β-glucan, glucose, fructose, pentosans, saccharose, kestose, neokestose, bifurcose, neobifurcose, acid galactoarabinoxylan, *etc*	β-glucan is the most important component because it is a constituent of the dietary fibre obtained from oats. β-glucan has important functional and nutritional properties, and exhibits a high viscosity at relatively low concentrations	[52, 53]
Lipids	Oat lipids are highly unsaturated and contain several essential fatty acids	Oats, after corn, have the highest lipid content of any cereal. Oat lipids include very high levels of antioxidants	[54, 55]
Antioxidants	Vitamin E (tocols), phytic acid, phenolic compounds, avenanthramides, flavonoids, and sterols	Antioxidants may reduce serum cholesterol concentrations, and inhibit the growth of certain cancer cells	[55]

Table 2 Health benefits of oats

Effect	Findings	Ref.
Hypocholesterolemic	An effect derived from β-glucan content, and demonstrated in normal and hypercholesterolemic subjects. The statistical significance of this cholesterol reduction has been variable, and remains controversial	[15, 18]
Hypoglycaemic	Studies have suggested that oat consumption can significantly decrease insulin response, fasting blood glucose levels, and the incidence of postprandial hyperglycaemia. However, some studies have failed to identify a diet-related effect on glycaemic control or a person's insulinemic response to oat-enriched products	[56-58]
Prevention of cancer	Selenium, present in oats, is involved in DNA repair and associated with a reduced risk for cancer; especially colon cancer. Furthermore, it is found in foods with a high fibre content	[59-61]
Reduction of hypertension	Soluble fibre-rich whole oats may be effective when consumed as dietary therapy for the prevention and adjunct treatment of hypertension	[62]
Immunomodulatory	β-glucans act by stimulating the immune system and inhibiting the growth of various bacteria, viruses, fungi, and parasites	[63]
Antioxidant	Oats contain chemicals with potential antioxidant properties; *e.g.*, vitamin E (tocols), phytic acid, and phenolic compounds, *etc*	[55]
Antiatherogenic	*In vivo* studies of atherosclerosis showed that oat bran reduced plasma cholesterol levels. However, it was difficult to determine whether its antiatherogenic effect was a result of reduced plasma cholesterol alone, or if additional effects of other oat components contributed to the result	[64]
Obesity control	Studies revealed that oats effectively reduced obesity, as well as indexes of serum lipid levels and liver function. These effects were observed when using β-glucan with the proper molecular weight	[14, 52]

disulfide-linked aggregates[21]. Similar to other cereal prolamins, the avenin polypeptides in oats tend to be rich in proline and glutamine, and the protein regions enriched in these two amino acids are associated with elicitation of CD. However, when compared to prolamins in other cereal grains, oat prolamins show the following differences in their molecular size, percentage, and amino acid content: (1) prolamins account for 10%-20% of the total protein in oats, compared to 40%-50% of the total protein in wheat[22,23]; (2) among the accepted prolamins, those found in maize, sorghum, and rice generally have the lowest contents of proline and glutamine (25%-30%), while prolamines in the Triticeae tribe (wheat, barley, and rye) can have proline plus glutamine contents that exceed 70% of their total amino acids. In contrast, proline and glutamine generally comprise 35%-50% of amino acids found in the prolamins of oats[24]; (3) in contrast to the single longer repetitive domain found in Triticeae prolamins, oat avenins contain two shorter domains with high contents of proline and glutamine[24]; and (4) the disulfide pattern in oat prolamins is different from those reported in wheat γ-gliadins[25] and low molecular weight (LMW)-glutenins[26]. In particular, the tandem cysteines at positions 145-146 in oat prolamins form a disulfide bond. This is in contrast to wheat proteins, where the two tandem cysteines are bonded to more distant cysteines within the prolamin[24].

Despite these reported differences, the avenins have not been well studied. As a result, the complete avenin genes described in current genetic databases represent only a few genotypes, and the variability displayed by avenin genes in oats is not well represented[21].

Clinical studies

The inclusion of oats in gluten-free foods is controversial, as previous studies have shown contradictory results regarding their toxicity. Janatuinen *et al*[27] conducted the first controlled study on the toxic effects of oats in CD patients, and since that time several other similar investigations have been conducted. Some researchers have claimed that celiac patients can consume oats and show no signs of intestinal inflammation[12,28-31]. In a study conducted by Størsrud *et al*[32,33], a small number of adult celiac subjects consumed pure oats (93 g/d) for 2 years with no reported adverse effects. The same

researchers also conducted a study in which a group of celiac children ingested a median of 43 g (up to 81 g/d) of oats daily for 2 years[34] with no adverse effects. Moreover, a randomized double-blind study conducted with newly diagnosed CD children showed that consumption of an oat-containing GFD for 1 year did not interfere with their clinical, serological or small bowel mucosal recovery. However, despite those results, 26% of the children in the oat-containing GFD group withdrew from that study for unknown reasons[34,35].

While the previously mentioned studies appear to support the safety of oat consumption by celiac patients, the results of other studies suggest that regular consumption of certain types of oats may be impossible for such patients, due to their toxic effects. Those studies revealed that oats can trigger an immune reaction in celiac patients[28,36-38] that results in activation of mucosal T-cells, subsequent gut inflammation, and eventual villous atrophy[37]. In those patients, the immune response against avenins may have been triggered by a mechanism similar to that which triggers a response to gluten contained in wheat, rye, or barley. Lundin *et al*[36] studied 19 celiac patients who consumed 50 grams of oats/day for 12 wk, and found that one patient was oat sensitive. CD patients have circulating anti-avenin antibodies[39,40], and a recent study revealed that dietary oats can alter the mRNA immune status of intestinal mucosa cells; suggesting T-cell activation and the presence of leaky tight-junctions[41]. Such findings indicate the need to distinguish between groups of celiac patients based on their sensitivity to different cereal grains, and also to identify the source of immunogenicity in avenin peptides.

Gluten contamination of commercial oat products

The phrase "pure oats" is used to describe oats that after being analysed using current test methods, appear to be uncontaminated with gluten from other closely related cereal grains, such as wheat, barley, and rye. However, the differences in the oat products used and the testing and reporting of the purity of oats further limited a comprehensive safety assessment. When studying literature reports, the study design or protocol did not always clearly describe the specifications used for defining "pure and uncontaminated" oats. While the most recent reports usually indicate whether the oats used in a particular study were tested for purity, many studies fail to indicate the lower limit of detection for their testing techniques or the cut-off values used when reporting that oat samples were free of gluten from other cereal grains.

According to the Codex Standard for food for special dietary use by persons intolerant to gluten, CODEX STAN118-1979 (revised 2008, http://www.foedevarestyrelsen.dk/SiteCollectionDocuments/25_PDF_word_filer%20til%20download/07kontor/Maerkning/Codex%20standard%20for%20gluten.pdf), oats can be tolerated by most but not all people who are intolerant to gluten. Therefore, whether oats that are not contaminated with wheat, rye or barley and are contained in foods covered by this standard can be considered safe for consumption by celiac patients may eventually be determined at the national level. Moreover, according to Commission Regulation (EC) No 41/2009 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:016:0003:0005:EN:PDF), which addresses the composition and labelling of foodstuffs suitable for people intolerant to gluten, the risk that oats may become contaminated with wheat, rye or barley during grain harvesting, transport, storage or processing remains a major concern. Therefore, the risk that oat-containing products might be contaminated with exogenous gluten should be taken into consideration when creating their labels.

Some studies have utilized the R5 ELISA method to determine the level of contamination in wheat, barley, or rye in oat products[42-44]. Koerner *et al*[43] used this method to confirm that the commercial oat supply in Canada is heavily contaminated with gluten from other grains. For example, about 88% of the tested oat samples (n = 133) showed a gluten level > 20 mg/kg. However, the problem with using this method is that the R5 antibody can react with certain types of pure oat seed[45]; hence, a test result suggesting "suspected contamination" from exogenous toxic cereal grains may not be real, due the R5 antibody reacting with certain amino acid sequences in the native oat proteins.

Diversity in potential immunogenicity depends on oat cultivars

Differences in the type of oat grain, oat purity, study design, as well as the specifications for gluten-free products in different countries, are some reasons why the current studies have not clearly established whether or not oats can be safely consumed by all celiac patients. These apparent contradictions might be explained by the fact that the oat varieties used in the diverse studies were different in regards to their prolamin genes, protein amino acid sequences, and the immunoreactivities of their toxic prolamins[46,47].

Our research group conducted a study using nine different varieties of oats obtained from various Australian and Spanish commercial sources, and demonstrated that oat immunogenicity varies depending on the cultivar used[45]. The oat grains were carefully inspected, controlled to maintain purity, and shown to be free of contamination. An analysis of DNA amplification products confirmed that the oat samples were not contaminated with wheat, barley, rye, or any mixture of these grains. The toxicity of each oat variety was evaluated using a moAb G12 immunoassay. The antibody used in the assay was obtained from the

α-2 gliadin 33-mer peptide, which is one of the most toxic peptides for CD patients. The nine varieties of oats were classified into three groups (high reactivity, intermediate activity, and no reactivity) based on their moAb G12 reactivity. We found that reactivity with the anti-33-mer moAb shown by the different oat varieties was correlated with T-cell proliferation and interferon gamma production by blood T-cells isolated from CD patients. These results suggest that a moAb G12-based immunotechnique may be a pragmatic method for evaluating the potential immunotoxicity of commercial cereals and grains[45,48].

Subsequent studies confirmed a direct correlation between the immunogenicity of the different varieties of oats and the presence of specific peptides with higher/lower potential immunotoxicity. This finding may explain why certain varieties of oats produce toxic effects when consumed by celiac patients, while others produce no adverse effects[21,49]. Moreover, oat peptides obtained from toxic cultivars have showed to differentially stimulate bona fide circulating dendritic cells obtained from celiac patients.

While inclusion of oats in a GFD might be beneficial due to their nutritional and health benefits, the source of the oats used and the cultivar selected are important factors to be considered. These factors must also be taken into account when developing food safety regulations, labelling oat-containing products as gluten-free, and designing clinical trials to study the effect of oats in celiac patients.

CONCLUSION

In summary, oats possess a variety of pharmacological activities and may exert antioxidant, anti-inflammatory, antidiabetic, and anticholesterolaemic effects. These properties have led to their wider use in human food. Inclusion of oats in a gluten-free diet might be valuable due to their nutritional and health benefits, and several countries currently permit oats to be included as an ingredient in such diets. However, it is extremely important to remember that *in vitro* studies have shown that the immunogenicity of oats varies depending on the cultivar used. Future clinical studies should be directed to the development of clinical trials with varieties previously identified as safe by reliable *in vitro* methods, such as moAb G12-based immunotechniques.

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MINIREVIEWS

Preoperative endoscopic diagnosis of superficial non-ampullary duodenal epithelial tumors, including magnifying endoscopy

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Abstract

Superficial non-ampullary duodenal epithelial tumor (SNADET) is defined as a sporadic tumor that is confined to the mucosa or submucosa that does not arise from Vater's papilla, and it includes adenoma and adenocarcinoma. Recent developments in endoscopic technology, such as high-resolution endoscopy and image-enhanced endoscopy, may increase the chances of detecting SNADET lesions. However, because SNADET is rare, little is known about its preoperative endoscopic diagnosis. The use of endoscopic resection for SNADET, which has no risk of metastasis, is increasing, but the incidence of complications, such as perforation, is significantly higher than in any other part of the digestive tract. A preoperative diagnosis is required to distinguish between lesions that should be followed up and those that require treatment. Retrospective studies have revealed certain endoscopic findings that suggest malignancy. In recent years, several new imaging modalities have been developed and explored for real-time diagnosis of these lesion types. Establishing an endoscopic diagnostic tool to differentiate between adenoma and adenocarcinoma in SNADET lesions is required to select the most appropriate treatment. This review describes the current state of knowledge about preoperative endoscopic diagnosis of SNADETs, such as duodenal adenoma and duodenal adenocarcinoma. Newer endoscopic techniques, including magnifying endoscopy, may help to guide these diagnostics, but their additional advantages remain unclear, and further studies are required to clarify these issues.

Key words: Endoscopy; Duodenoscopy; Duodenal neoplasms; Narrow band imaging; Pathology

Core tip: Because superficial non-ampullary duodenal epithelial tumor is rare, a preoperative endoscopic diagnostic technique to differentiate between adenoma and adenocarcinoma has not yet been established. Recently, many new imaging modalities have been developed and explored for use in the real-time diagnosis of these types of lesions. Newer endoscopic techniques, including magnifying endoscopy, may help to guide these diagnostics, but their additional advantages remain unclear, and further studies are required to clarify these issues.

Tsuji S, Doyama H, Tsuji K, Tsuyama S, Tominaga K, Yoshida N, Takemura K, Yamada S, Niwa H, Katayanagi K, Kurumaya H, Okada T. Preoperative endoscopic diagnosis of superficial non-ampullary duodenal epithelial tumors, including magnifying endoscopy. *World J Gastroenterol* 2015; 21(41): 11832-11841 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i41/11832.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i41.11832

INTRODUCTION

Epithelial tumors of the duodenum are relatively rare[1], with primary duodenal carcinomas comprising only approximately 0.5% of malignant gastrointestinal tumors[2]. Duodenal adenomas are uncommon lesions with a reported prevalence of less than 0.4% in patients undergoing esophago-gastro-duodenoscopy[3,4]. Surgical treatment of non-ampullary duodenal tumors can be invasive because of anatomical complexities. Recent developments in endoscopic technology, such as high-resolution endoscopy and image-enhanced endoscopy, may increase the chances of detecting superficial non-ampullary duodenal epithelial tumor (SNADET) lesions and allow their resection without surgery[5,6]. The prognosis of patients with advanced duodenal carcinomas is poor[7], and early detection and treatment are essential.

Endoscopic resection (ER) is a minimally invasive, local treatment that can be used in cases of SNADET with no risk of metastasis[8]. However, the incidence of complications, such as perforation, that are associated with the use of ER to treat SNADET is significantly higher than in any other part of the digestive tract[6,9,10] because of the thinness of the duodenal wall and its exposure to bile and pancreatic juice[9,11,12]. A preoperative diagnosis is required to distinguish between lesions that should be followed up and those that require treatment. Follow-up without ER for low-grade adenoma (LGA) is acceptable because its risk of progression to cancer is approximately 5%[9,13]. However, because SNADET is rare, much remains unknown about its preoperative endoscopic diagnosis.

SNADET is defined as a sporadic tumor that is confined to the mucosa or submucosa that does not arise from Vater's papilla, and it includes adenoma and adenocarcinoma. This review focuses on the present status of the preoperative endoscopic diagnosis of SNADETs.

HISTOPATHOLOGICAL DIAGNOSES REFERRED TO THE REVISED VIENNA CLASSIFICATION AND CLINICAL MANAGEMENT

Recently, a new set of categories for classifying gastrointestinal neoplasias (*i.e.,* the Vienna classification) has been proposed (Table 1) to bridge the East-West gap[14]. Adenomas of the gastrointestinal tract can be categorized as LGA (category 3) and high-grade dysplasia (HGD; category 4.1), according to the diagnostic classification of dysplasia established in the revised Vienna classification. Several previous studies[13,15,16] have classified histopathological diagnoses of SNADETs based on the revised Vienna classification. For the purposes of these studies, LGA was included in the revised Vienna Category 3 (C3), and HGD and superficial adenocarcinoma were included in the revised Vienna Category 4 (C4), such that all C3 lesions were non-malignant, and all C4 lesions were classified as cancer. In this review, only LGA lesions are considered to be sporadic non-ampullary adenomas because LGA lesions show a low risk of progression to adenocarcinoma[9,13], and non-ampullary duodenal cancers are also considered to be C4 lesions.

The choice of treatment depends on the overall size of a lesion; the depth of its invasion as assessed endoscopically, radiologically, or ultrasonographically; and general factors, such as a patient's age and comorbid conditions. For gastric, esophageal, and non-polypoid colorectal carcinomas that are well differentiated or moderately differentiated and show only minimal submucosal invasion (sm1) without lymphatic involvement, local resection is sufficient. Likewise, for polypoid colorectal carcinomas with deeper submucosal invasion in the stalk/base but without lymphatic or blood vessel invasion, complete local resection is considered adequate treatment[14,17].

DEFINITION OF SPORADIC NON-AMPULLARY ADENOMA

Duodenal adenomas that do not involve the major duodenal papilla are characterized as benign epithelial tumors of the small bowel. They may occur sporadically or in the context of genetic syndromes, such as familial adenomatous polyposis or Peutz-Jeghers syndrome. A sporadic non-ampullary adenoma is regarded as a precancerous lesion. Previous reports have suggested that there are two carcinogenesis pathways of duodenal cancer: the adenoma-carcinoma sequence and the development of *de novo* cancer[18-20]. Sporadic non-ampullary adenoma should be differentiated

Table 1 The revised Vienna classification and clinical management

Category	Diagnosis	Clinical management
1	Negative for neoplasia	Optional follow-up
2	Indefinite for neoplasia	Follow-up
3	Mucosal low-grade neoplasia Low-grade adenoma Low-grade dysplasia	Endoscopic resection or follow-up
4	Mucosal high-grade neoplasia 4.1 High-grade adenoma/ dysplasia 4.2 Noninvasive carcinoma (carcinoma in situ) 4.3 Suspicious for invasive carcinoma 4.4 Intramucosal carcinoma	Endoscopic or surgical local resection
5	Submucosal invasion by carcinoma	Surgical resection

Table 2 Relationship between endoscopic findings and final histological grade

	Category3 (n = 121)		Category4 (n = 275)		P value
Diameter (mean, mm)	11.5 ± 0.7		17.5 ± 0.7		< 0.0001
Location (portion)					
First	23	19%	46	17%	NS
Second	92	76%	205	74%	
Third or fourth	6	5%	24	9%	
Color					
Red	36	30%	124	45%	< 0.01
Isochromatic or white	85	70%	151	55%	
Macroscopic type					
0-Ⅰ	29	24%	58	21%	NS
0-Ⅱa	71	59%	170	62%	
0-Ⅱc	21	17%	47	17%	

Color or macroscopic type is adopted from the predominant color when tumor showed multiple colors or macroscopic types. Data from Goda *et al*[5]. NS: Not significant.

from polyps that occur in genetic syndromes or at the papilla. Polyps are associated with an increased risk of malignancy, and they require different diagnostic and therapeutic strategies than those for sporadic non-ampullary adenomas[21,22]. Sporadic non-ampullary adenomas account for up to 7% of duodenal polyps that are biopsied using upper endoscopy, which is a prevalence of 1-3 cases per 1000[3,23]. The mean age at diagnosis is usually in the seventh decade, and the incidence is approximately equal among men and women. The majority of patients are asymptomatic at the time of diagnosis[24].

DEFINITION OF EARLY NON-AMPULLARY DUODENAL CANCER

Owing to the low prevalence of SNADET, there is no established definition for early non-ampullary duodenal cancer regarding its depth of invasion and risk of lymph node metastasis[8]. Previous studies have followed the rules that are used for early colorectal[25] or gastric cancer[26] and for tumor invasion into the lamina propria, muscularis mucosa (T1a) or submucosa (T1b), regardless of lymph node metastasis[18,27,28]. There is little information regarding the pathological risk factors for lymph node metastasis of T1a and T1b in non-ampullary duodenal cancer. Nagatani *et al*[29] found no incidence of lymph node metastasis among 40 pT1a cancers, while Fujisawa *et al*[27] reported no metastasis among 166 pT1a cancers. The incidence of lymph node metastasis among pT1b cancers was reported to be 5.3%-5.4%[27,28].

DIFFERENTIAL DIAGNOSIS BETWEEN SNADET C3 AND C4 LESIONS

Characterization using conventional white light imaging

C3 lesions are usually solitary and sessile; and although they can be located in any part of the duodenum, they are found distally in the majority of patients[3]. Both C3 and C4 lesions arise most frequently in the second portion of the duodenum, especially in the periampullary area[18,30,31].

In a Japanese multicenter study, the mean tumor diameter of C4 lesions was significantly larger than that of C3 lesions. C4 lesions were solitary or showed a predominantly red color significantly more frequently than C3 lesions. There were no significant differences between final histological grade and other endoscopic findings, such as tumor location and macroscopic type (Table 2)[5]. Okada *et al*[13] showed that a lesion diameter of ⩾ 20 mm was significantly predictive of progression to adenocarcinoma. A tumor diameter > 5 mm also seemed indicative for C4 lesion tumors, and this might suggest a recent increase in the number of small C4 lesions of 6-10 mm in diameter[5]. In addition, out of 139 SNADETs, this case series found 46 mucosal carcinomas (33%) and one submucosal carcinoma that had a tumor diameter of 6-10 mm[5]. Lesions with a depression component also tended to have a higher cancerous component[32,33]. Endoscopic features of C4 lesions included a red color in the tumor and a nodular, rough surface[27,32].

Whitish villus, milk-white mucosa, and white opaque substance

Inatsuchi *et al*[34] reported that 84% of SNADETs had a whitish villus, which may be helpful in recognizing these lesions under conventional endoscopy. Yoshimura *et al*[15] showed that 92% of SNADETs had a milk-white mucosa on conventional endoscopy, which is a common endoscopic finding for C3 and C4 lesions. A white opaque substance (WOS) was reported first by Yao *et al*[35] as a substance in the superficial area of a gastric neoplasia that is visualized in magnifying endoscopy with narrow-band imaging (M-NBI). WOS represents intramucosal accumulation of lipid droplets using oil red O staining[36]. Tanaka *et al*[37] suggested

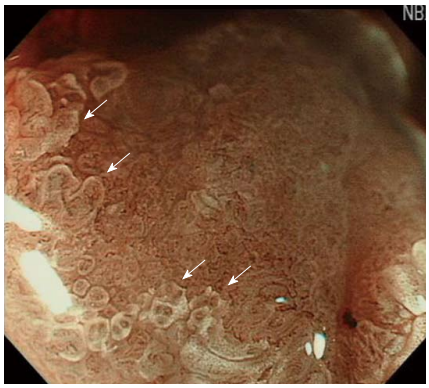

Figure 1 Duodenal adenocarcinoma imaged with magnifying endoscopy with narrow-band imaging. White opaque substance (WOS) in lesion margins on magnifying endoscopy with narrow-band imaging (M-NBI). Speckled WOS is found at the lesion margins (arrows), and little is in the central area.

Table 3 Comparison of magnifying endoscopy with narrow-band imaging findings according to vessel plus surface classification system and final histological grade in all 64 superficial non-ampullary duodenal epithelial tumors

	Diagnosis from ER specimens				*P* value
	Category 3 (*n* = 27)		Category 4 (*n* = 37)		
Demarcation line	27	100%	37	100%	1
Microvascular pattern; V					
Regular/Absent	10/8	37%/30%	5/17	14%/46%	0.56
Irregular	9	33%	15	41%	
Microsurface pattern; S					
Regular	13	48%	4	11%	0.0008
Irregular	14	52%	33	89%	

ER: Endoscopic resection.

that whitish villi were a result of lipids in epithelial cells at the villi tips. Whitish villus, milk-white mucosa, and WOS are thought to have the same appearance.

It has been reported that the distribution pattern of milk-white mucosa is classified as either entire or marginal, and the frequency of the marginal type of milk-white mucosa (Figure 1) is significantly higher in C4 lesions compared to C3 lesions[15]. Whitish villus, milk-white mucosa, and WOS are characteristic of SNADETs, and their individual characteristics may also be useful in differentiating between C3 and C4 lesions.

Characterization using magnifying endoscopy with NBI

NBI is an innovative optical image-enhancing technology that uses narrow blue and green wavelengths to increase the conspicuity of vessels[38]. M-NBI enables clear visualization of superficial microanatomy and can be used to differentiate between cancerous and non-cancerous lesions of the digestive tract more accurately than conventional endoscopy[39-44]. However, there have been only a few reports characterizing SNADET using M-NBI.

Yoshimura *et al*[15] showed that the frequency of a microvascular pattern network type was significantly higher in C4 lesions. Recently, Kikuchi *et al*[16] have proposed a diagnostic algorithm of M-NBI for SNADET, as shown in Figure 2. They defined vessels that were dilated, tortuous, or had irregular diameter, size, or shape as having an "unclassified pattern"; all C4 lesions had this pattern[16]. In previous studies, the frequency of an ill-defined mucosal pattern (Figure 3) and mixed-type lesions with multiple surface patterns (Figure 4) were distinctive findings in C4 lesions[15,16].

Vessel plus surface classification system for magnifying endoscopy with narrow-band imaging

Between December 2008 and January 2015, we retrospectively used ER to investigate both the endoscopic findings and the resected specimens of 64 SNADETs at our hospital. We used the established vessel plus surface (VS) classification system and M-NBI to diagnose early gastric cancer[41], which is the most commonly used system in clinical practice[42].

We determined whether there was a demarcation line (DL) between a lesion and the background mucosa. Microvascular (MV) patterns and microsurface (MS) patterns were categorized as regular, irregular, or absent. Lesions presenting with an irregular MV pattern with a DL and/or an irregular MS pattern with a DL were diagnosed as cancerous (C4)[42].

Table 3 shows a comparison of the M-NBI findings for the 64 lesions based on the VS classification. DLs were observed in all of the lesions (100%). There was no significant difference in MV patterns between the C3 and C4 groups. In the SNADETs, there was a tendency for irregular MV patterns to be observed in C3 and C4 lesions. More than 90% of all of the SNADETs in this study demonstrated WOS in the superficial parts of the lesions, obscuring the morphology of subepithelial microvessels in approximately 40% of all lesions. One explanation might be that WOS made it difficult to evaluate the overall distribution and arrangement of microvessels. An irregular MS pattern was present in 14 lesions (52%) in the C3 group and in 33 lesions (89%) in the C4 group, indicating a significant intergroup difference (P = 0.0008). An irregular MS pattern was a reliable marker for differentiating between benign and malignant gastric lesions[40]. Typical cases in the C3 and C4 groups where M-NBI findings were useful for distinguishing between C3 and C4 are shown in Figure 5A-C (C3) and in Figure 6A-C (C4). False-positive cases characterized by malignant M-NBI diagnoses and benign pathological diagnoses are shown in Figure 7A-C. We found that an irregular MS pattern was significantly more frequent in the C4 group, while there was no significant difference in MV patterns between the C3 and C4 groups. These findings may be useful in distinguishing between carcinomas and benign lesions in SNADETs. However, the additional advantages of M-NBI remain unclear, and further studies, including ones on the relationship between histopathological type and MS findings, are

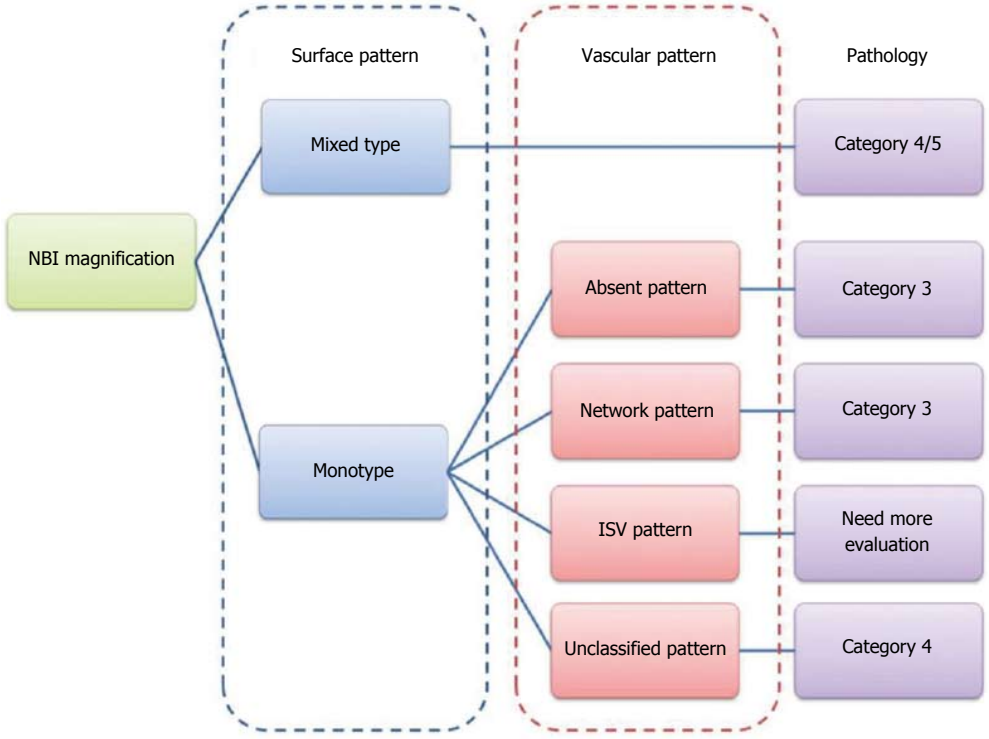

Figure 2 Diagnostic algorithm of magnifying endoscopy with narrow band imaging for superficial non-ampullary duodenal epithelial tumor. From Kikuchi *et al*[16].

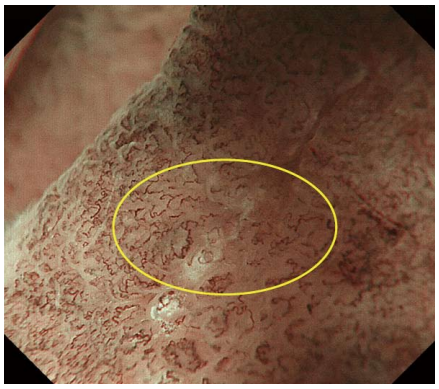

Figure 3 Duodenal adenocarcinoma imaged with magnifying endoscopy with narrow-band imaging. An indistinct area of a marginal crypt epithelium (MCE) structure as imaged by magnifying endoscopy with narrow-band imaging (M-NBI). There are no discernible microsurface features (yellow circle).

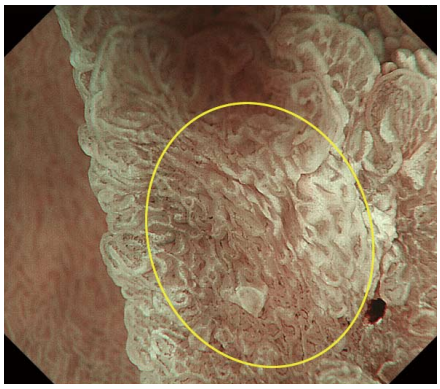

Figure 4 Duodenal adenocarcinoma imaged with magnifying endoscopy with narrow-band imaging. Because of uneven distribution of white opaque substance (WOS) on magnifying endoscopy with narrow-band imaging (M-NBI), this lesion displays multiple microsurface patterns as mixed-type (yellow circle).

required to clarify these issues.

Magnifying chromoendoscopy

Chromoendoscopy was introduced to improve the success of duodenal polyp detection and differentiation[45,46]. Chromoendoscopy in combination with magnifying endoscopy is useful in distinguishing neoplastic from non-neoplastic colorectal polyps[47]. It has been important to show that magnifying endoscopy combined with chromoendoscopy is useful to discriminate between neoplastic and non-neoplastic colonic polyps, based on the pit-pattern classification[48-51]. Endo *et al*[1,52] diagnosed patients with sporadic non-ampullary adenoma or non-ampullary duodenal cancer based on magnified images that were stained with crystal violet through the use of the pit-pattern classification for colonic mucosa. Using magnification endoscopy, they categorized SNADETs into convoluted, leaf-like, reticular/sulciolar, and colon-like patterns[1,52].

Preoperative diagnosis using biopsy

Okada *et al*[13] analyzed 68 sporadic non-ampullary duodenal adenomas that were diagnosed using biopsy and reported that LGA lesions show a low risk of progression to adenocarcinoma, whereas HGD lesions show a high risk of progression to adenocarcinoma. In a preoperative diagnosis, accurately differentiating

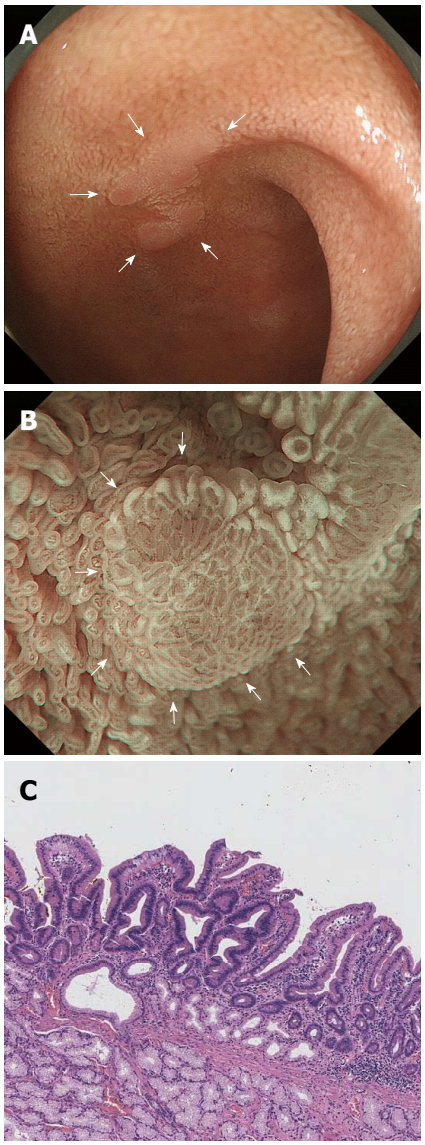

Figure 5 Magnifying endoscopy with narrow-band imaging imaging of a duodenal adenoma. A: Endoscopic findings using conventional endoscopy with white light imaging. A pale, slightly elevated lesion (10 mm in diameter, arrow) is observed in the proximal duodenum; B: Endoscopic findings using magnifying endoscopy with narrow-band imaging (M-NBI). A demarcation line (DL, arrows) separates changes in the mucosal microsurface (MS) structure from the surrounding normal mucosa. Vessel plus surface (VS) classifications: V, Because of the white opaque substance (WOS), the morphology of the subepithelial microvessels cannot be observed, making this an absent microvascular (MV) pattern; S, The WOS has a regular reticular pattern with a symmetrical distribution and regular arrangement. Thus, this lesion is graded as a regular MS pattern using WOS as a marker for the MS pattern. The VS classification of this lesion was absent MV pattern and regular MS pattern (WOS+) with a DL. Therefore, the M-NBI diagnosis was benign; C: The final histological diagnosis was of a low-grade adenoma.

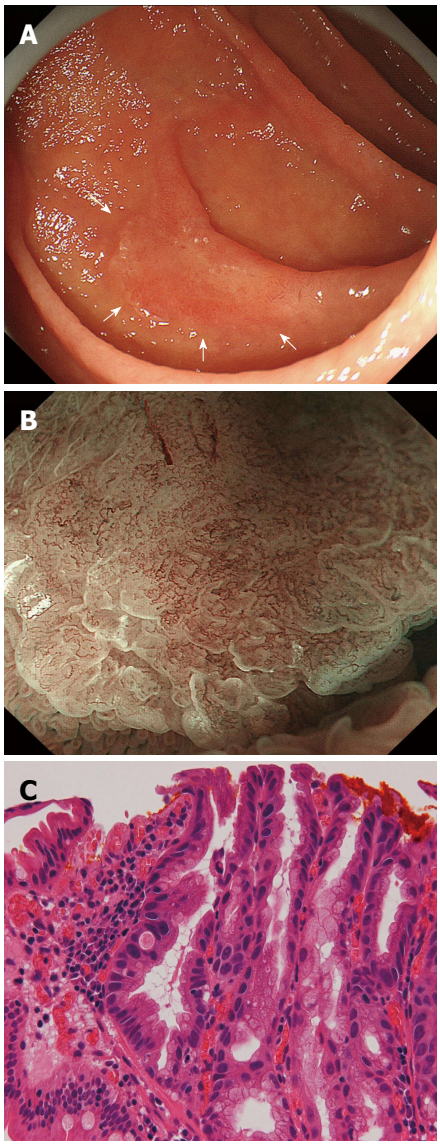

Figure 6 Duodenal adenocarcinoma with typical magnifying endoscopy with narrow-band imaging findings. A: Endoscopic findings using conventional endoscopy with white light imaging. A reddish, slightly elevated lesion (13 mm in diameter, arrows) is observed in the second portion of the duodenum; B: Endoscopic findings using magnifying endoscopy with narrow-band imaging findings (M-NBI). A clear demarcation line (DL) is visible because of differences in the vessel plus surface (VS) component between the cancerous and noncancerous mucosa. V: Proliferation of microvessels with variable sizes, asymmetrical distribution and irregular arrangement make this an irregular microvascular (MV) pattern; S: There are areas where the marginal crypt epithelium (MCE) cannot be visualized and where the visible MCE shows a variety of morphologies, an asymmetrical distribution and an irregular arrangement. This lesion is assessed as an irregular mucosal microsurface (MS) pattern. The VS classification of this lesion was an irregular MV pattern and irregular MS pattern with a DL. Therefore, the M-NBI diagnosis was cancer; C: The final histological diagnosis was a well-differentiated intramucosal adenocarcinoma.

cancer from adenoma is difficult based on biopsy findings alone. Forceps biopsy is recommended for all suspect lesions, although 15%-56% of cancers may be missed at biopsy due to sampling error compared with using surgically resected specimens[53,54]. In a multicenter study, the sensitivity, specificity and accuracy of preoperative diagnosis using biopsy for final HGD and superficial adenocarcinoma histology were 58%, 93%, and 68%, respectively[5]. In another study, T1a cancer was observed in 13.5% of patients in whom initial biopsies indicated simple adenomas[55]. Owing to the thinness of the duodenal wall, the biopsy procedure itself may induce unintended fibrosis

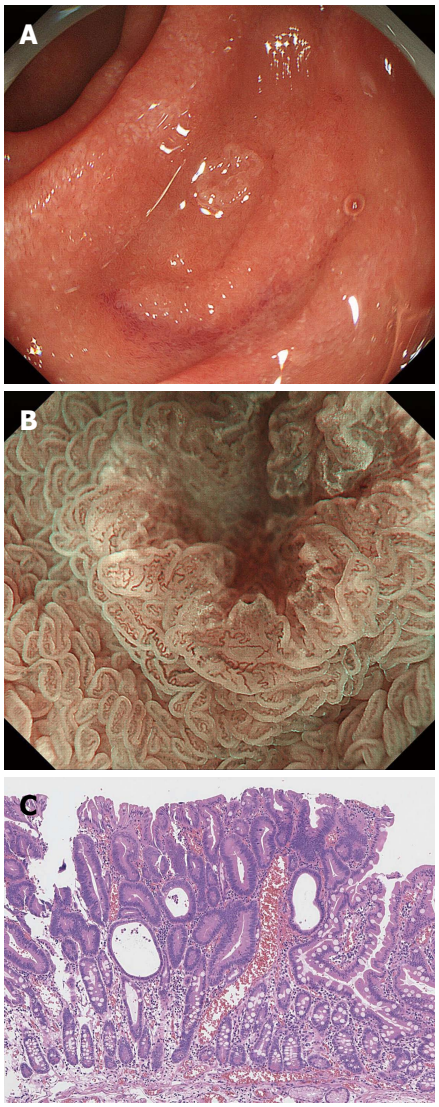

Figure 7 False-positive magnifying endoscopy with narrow-band imaging diagnosis. A: Endoscopic findings using conventional endoscopy with white light imaging. A whitish, slightly depressed lesion (5 mm in diameter) is observed in the second portion of the duodenum. In this case, magnifying endoscopy with narrow-band imaging diagnosis (M-NBI) examination was conducted before biopsy; B: Endoscopic findings using M-NBI. A clear demarcation line (DL) is visible because of differences in the vessel plus surface (VS) component between the tumor and surrounding mucosa. V: The individual vessels show a variety of morphologies, such as open- and closed-looped and coil-shaped, with no two microvessels sharing the same morphology. The microvessels are anastomosing with each other within the intervening parts but show no consistent regularity. Therefore, this lesion was assessed as an irregular microvascular (MV) pattern; S: This individual section of marginal crypt epithelium (MCE) shows a curved morphology but lacks continuity or a consistent directionality, and the intervening parts are also irregular with unequal sizes. Therefore, this lesion was assessed as an irregular mucosal microsurface (MS) pattern. The VS classification of this lesion was an irregular MV pattern and irregular MS pattern with a DL. Therefore, the M-NBI diagnosis was cancer; C: The final histological diagnosis was a low-grade adenoma.

associated with a lesion, which may complicate subsequent ER[10]. Consequently, it is necessary to perform a biopsy while causing a minimal amount of damage, and ER as a diagnostic therapy should be considered in some cases that are endoscopically diagnosed as carcinoma.

Confocal laser endomicroscopy and autofluorescence imaging

In recent years, many new imaging modalities have been developed and explored for use in the real-time diagnosis of duodenal lesions[56-58]. Confocal laser endomicroscopy (CLE) is a powerful technology that provides magnification × 1000 imaging using intravenous fluorescein as a contrast agent[59]. Currently, there are two types of CLE: probe-based CLE (pCLE) and endoscopic-based CLE (eCLE)[60]. In a recent study, pCLE was used along with NBI (GIF H-180; Olympus) for duodenal adenoma diagnosis, and it was concluded that pCLE provided better sensitivity than NBI (92% *vs* 83%, $P = 0.8$); duodenal adenoma diagnosis criteria for pCLE and NBI in this study were based on Barrett's esophagus criteria[58]. Pittayanon *et al*[61] reported that the diagnostic criteria for duodenal non-adenomatous and adenomatous lesions using pCLE were normal epithelium border with regular capillary pattern and dark/irregular/non-structural mucosa with normal or abnormal capillary networks, respectively. Autofluorescence imaging (AFI) is an endoscopic technique that uses autofluorescence that is emitted from an endogenous fluorophore following exposure to short-wavelength photoexcitation[62]. AFI has not been used to evaluate duodenal and periampullary lesions. Many new imaging modalities seem to be useful, but because of insufficient data on this uncommon entity, a large multicenter study is required to support this concept.

ENDOSCOPIC DIAGNOSIS OF SNADET EXTENT AND INVASION DEPTH

Determining SNADET margins using conventional endoscopy is easy, as it is similar to detecting epithelial tumors of the colon or rectum[1]. However, it is difficult to differentiate T1a from T1b non-ampullary duodenal cancer using barium studies or endoscopy[27]. Central dimpling or ulceration observed during endoscopy suggests invasive carcinoma[63]. Several previous studies have classified morphological types of superficial SNADETs based on the classification criteria that are used for colorectal tumors[20,27,28]. Macroscopic types based on endoscopic features include the protruded pedunculated (Ip), protruded sessile (Is), and semipedunculated (Isp) types and the superficial elevated (Ⅱa), flat (Ⅱb), and superficial shallow or depressed (Ⅱc) types[26]. Previous studies showed that 0-Ⅰ or 0-Ⅱa + Ⅱc macroscopic types with a red color were usually endoscopic features of submucosal carcinoma[5,29]. Endoscopic ultrasonography (EUS) is accurate in diagnosing gastrointestinal abnormalities because of its ability to image intestinal wall architecture and its surrounding structures in detail[64]. Tio *et al*[65] reported that EUS is accurate in diagnosing duodenal sessile villous adenomas, and it is, therefore, useful in planning treatment. EUS helps to evaluate larger lesions (greater than 2 cm

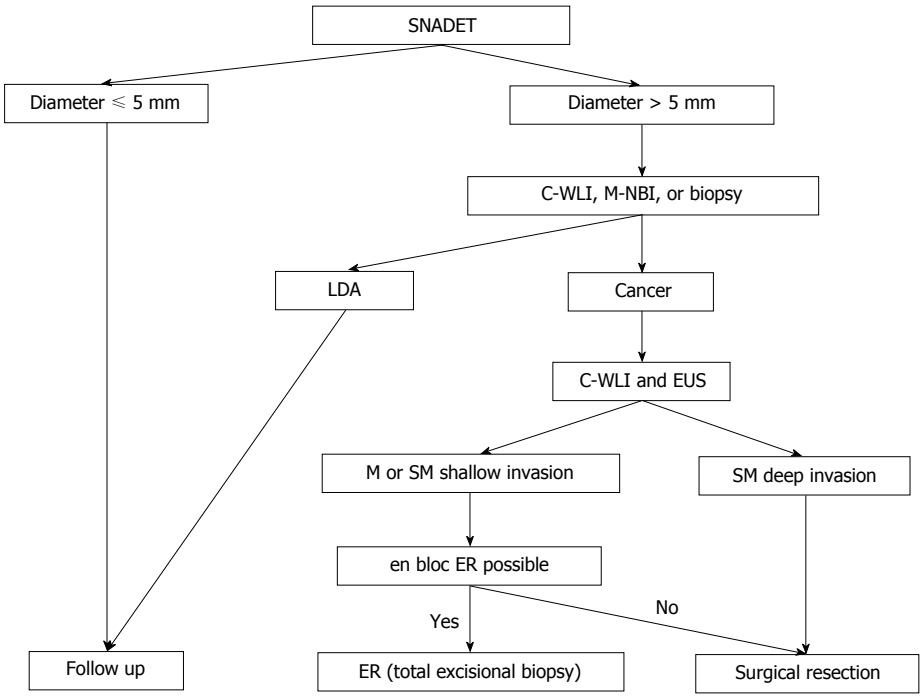

Figure 8 Suggested algorithm for the management of superficial non-ampullary duodenal epithelial tumor according to depth of invasion; tumor size; endoscopic findings, including magnifying endoscopy; and biopsy results. Endoscopic features of cancer are a red color in the tumor; a nodular, rough surface on conventional white light imaging; a marginal type of milk-white mucosa; an unclassified vascular pattern; a frequency of ill-defined mucosal pattern; and a population of mixed-type lesions with multiple surface patterns on magnifying endoscopy with narrow-band imaging. Endoscopic features of submucosal carcinoma are ulceration and a 0-Ⅰ or 0-Ⅱa + Ⅱc macroscopic type with a red color. SNADET: Superficial non-ampullary duodenal epithelial tumor; C-WLI: Conventional white-light imaging; M-NBI: Magnifying endoscopy with narrow-band imaging; LDA: Low-grade adenoma; EUS: Endoscopic ultrasonography; ER: Endoscopic resection.

in size) to establish the relationship of a duodenal polyp to the pancreatobiliary tree and to determine endoscopic resectability when biopsy specimens have shown HGD[66]. Preoperative EUS for six submucosal carcinomas enabled the prediction of submucosal invasion with 67% accuracy[5].

CONCLUSION

From this review, a suggested algorithm for the management of SNADET is shown in Figure 8. Given the heterogeneity of the lesions and the patient population, it is difficult to set guidelines that would encompass all possible scenarios, so each case must be taken on an individual basis. Because the incidence of SNADET is extremely rare, endoscopic findings that suggest early non-ampullary duodenal cancer have not yet been established. As indications for endoscopy increase and as techniques evolve, the rate of duodenal adenoma and duodenal adenocarcinoma detection, especially of small lesions, will likely increase. Newer endoscopic techniques, including magnifying endoscopy, may help to guide these diagnostics, but their additional advantages remain unclear, and further studies are required to clarify these issues.

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Endosonography guided management of pancreatic fluid collections

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Abstract

The revised Atlanta classification of acute pancreatitis was adopted by international consensus, and is based on actual local and systemic determinants of disease severity. The local determinant is pancreatic necrosis (sterile or infected), and the systemic determinant is organ failure. Local complications of pancreatitis can include acute peri-pancreatic fluid collection, acute necrotic collection, pseudocyst formation, and walled-off necrosis. Interventional endoscopic ultrasound (EUS) has been increasing utilized in managing these local complications. After performing a PubMed search, the authors manually applied pre-defined inclusion criteria or a filter to identify publications relevant to EUS and pancreatic collections (PFCs). The authors then reviewed the utility, efficacy, and risks associated with using therapeutic EUS and involved EUS devices in treating PFCs. Due to the development and regulatory approval of improved and novel endoscopic devices specifically designed for transmural drainage of fluid and necrotic debris (access and patency devices), the authors predict continuing evolution in the management of PFCs. We believe that EUS will become an indispensable part of procedures used to diagnose PFCs and perform image-guided interventions. After draining a PFC, the amount of tissue necrosis is the most important predictor of a successful outcome. Hence, it seems logical to classify these collections based on their percentage of necrotic component or debris present when viewed by imaging methods or EUS. Finally, the authors propose an algorithm for managing fluid collections based on their size, location, associated symptoms, internal echogenic patterns, and content.

Key words: Endoscopic ultrasound; Drainage; Pancreatic fluid collection; Pseudocyst; Patency device; Abscess; Walled of necrosis; Pancreas

Core tip: The revised Atlanta classification of acute pancreatitis was approved by international consensus, and is based on actual local and systemic determinants of disease severity. Local complications of pancreatitis can include acute peri-pancreatic fluid collection, acute necrotic collection, pseudocyst formation, and walled-off necrosis. Interventional endoscopic ultrasound (EUS) has been increasingly utilized in managing pancreatitis. This review describes the utility, efficacy, and risks associated with using therapeutic EUS and involved EUS devices to manage acute pancreatitis. The authors propose an algorithm for use in managing pancreatic fluid collections based on their size, location, associated symptoms, internal echogenic patterns, and content.

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INTRODUCTION

In 1992, the Atlanta classification of acute pancreatitis was adopted by international consensus. While the Atlanta classification attempted to standardize reporting and communication among health care professionals, some of the terminology used was confusing and failed to objectively describe complications associated with pancreatitis. This made it difficult to provide proper treatment. A revised Atlanta classification was released in 2012 after years of web-based consultation among a global panel of experts and international pancreatic associations[1-3]. The new classification system is based on actual local and systemic determinants of severity, rather than descriptions of events correlated with severity[1,2]. The local determinant is pancreatic necrosis (sterile or infected) and the systemic determinant is organ failure (transient or persistent). Acute pancreatitis is now classified into two phases (early and late), and its severity is classified as mild, moderate or severe. Mild acute pancreatitis is not accompanied by organ failure, local or systemic complications, and usually resolves in the first week. Moderately severe acute pancreatitis is accompanied by transient organ failure, as well as local complications or exacerbation of a co-morbid disease. Severe acute pancreatitis presents with persistent organ failure (> 48 h duration) (Table 1).

Local complications of pancreatitis can include acute peri-pancreatic fluid accumulation, acute necrotic collection (ANC, sterile or infected), pseudocyst formation, and development of walled-off necrosis (WON) (sterile or infected). WON is characterized by a distinct rim that forms around areas of tissue necrosis and adjacent pancreatic parenchyma. Interventional endoscopic ultrasound (EUS) has been increasing utilized to help manage these local complications[4,5]. To gather information for this review, the authors searched English language medical literature and reviewed articles which described the utility, efficacy, and risks associated with using therapeutic EUS and its involved devices in these clinical settings, which we grouped together as pancreatic fluid collections (PFCs). The authors propose an algorithm for use in managing pancreatic fluid collections based on their size, location, associated symptoms, internal echogenic pattern, and structure.

METHODS AND REVIEW STRATEGY

On November 15, 2014, the authors performed a PubMed search using the abbreviation EUS in combination with phrases or words related to pancreatic fluid collection; such as pseudocyst, fluid collection, abscess, and WON. Next, pre-defined inclusion criteria or filters were manually applied to the PubMed search results (Figure 1). The inclusion criteria were: (1) original report; (2) case number > 6; and (3) English language publication only. The authors then manually reviewed the publications and their listed references, *i.e.,* cross-reference search. Finally, each published paper was jointly reviewed by two authors of this review article, and relevant important information was extracted.

INDICATIONS AND TIMING FOR TRANSMURAL DRAINAGE

The decision to drain a pancreatic fluid collection depends on several factors, including the patient's clinical condition and symptoms, the change in amount of accumulated fluid over time, the time from the onset of symptoms, and the presence of infection or other complications. Asymptomatic pancreatic and/or extra-pancreatic fluid collections do not warrant intervention regardless of their size, location, and/or extension. Instead, drainage is considered only when a fluid collection causes clinical symptoms or displays signs of infection[6]. Infection is most common in fluid surrounding necrotic tissue, and is suggested by the presence of air pockets inside the accumulation and visible on a computerized tomography (CT) scan. If a clinical scenario strongly suggests an infected fluid collection, it can be verified by performing and examining the contents of a fine needle aspiration. Patients with sterile accumulations, luminal or biliary

Table 1 Revised Atlanta classification (2012) of pancreatic/peripancreatic fluid collections

Type of pancreatic or peripancreatic fluid collection	Etiology	Capsule	Specific features
Acute peripancreatic fluid collection, APFC	≤ 4 wk after onset of acute interstitial-edematous pancreatitis	-	Homogeneous, liquid, infection +/-, no features of a pseudocyst, usually resolves spontaneously
Pancreatic pseudocyst, PPC	> 4 wk after onset of acute interstitial-edematous pancreatitis	+	Round/oval, Liquid, no non-liquid contents, persistent
Acute necrotic collection, ANC	Acute necrotizing pancreatitis	-	Heterogeneous, liquid and necrotic contents, usually resolves spontaneously
Walled-off pancreatic necrosis, WOPN	> 4 wk after onset of necrotizing pancreatitis	+	Heterogeneous, liquid and necrotic contents, infection +/-

ANC: Acute necrotic collection.

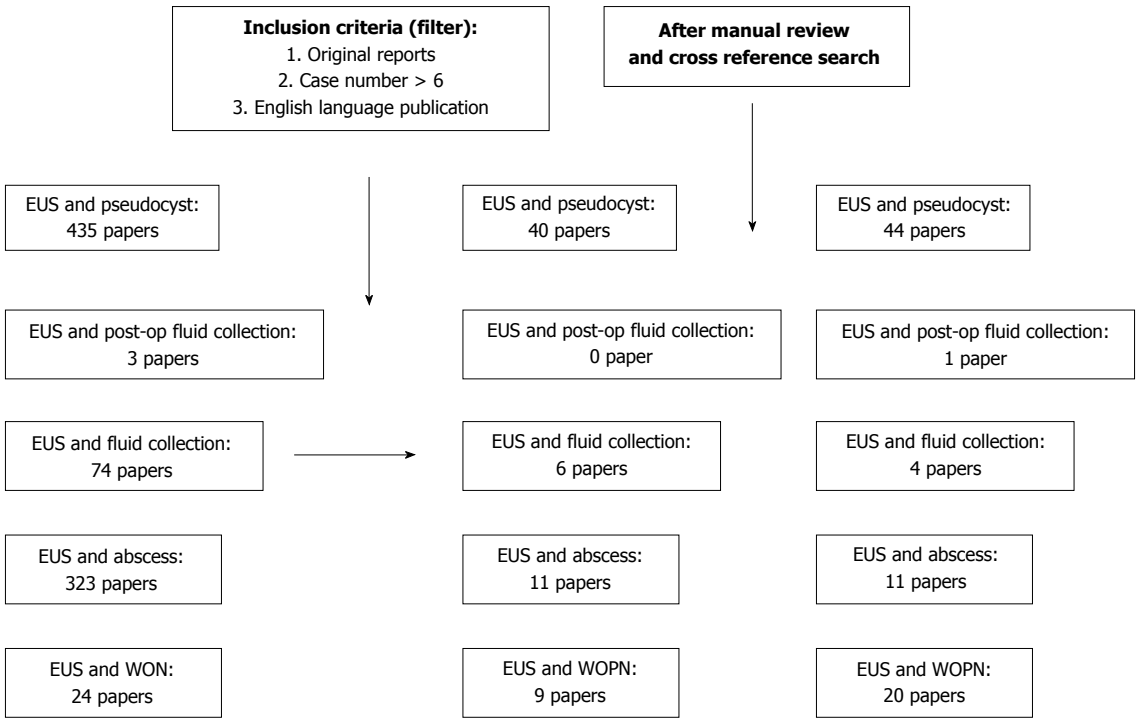

Figure 1 On November 15, 2014, the authors performed a PubMed search using following key word sets: endoscopic ultrasound in combination with terminologies related to pancreatic fluid collections such as pseudocyst, fluid collection, abscess, and walled off necrosis. Each published paper was simultaneously reviewed by two authors who extracted important information related to this review. EUS: Endoscopic ultrasound; WON: Walled off necrosis.

obstruction resulting from external compression, persistent abdominal pain requiring narcotics, or an undiagnosed sepsis syndrome should receive drainage[7]. PFCs can be drained using surgical, percutaneous or endoscopic methods.

Proper timing is critical for successful endoscopic drainage in cases of necrotizing pancreatitis. Interventions made within the first several weeks of necrotizing pancreatitis generally lead to poor outcomes. The guiding principle for timing of debridement is to delay any intervention until the collection has become encapsulated and liquefied as much as possible. Encapsulation does not usually occur until at least 4 wk after the initial injury.

Endoscopic methods for draining collected fluid have shown efficacies comparable to those achieved using surgical methods. Furthermore, endoscopic treatments usually result in shorter hospital stays, better patient physical and mental health, and lower treatment costs compared to surgery[8]. Percutaneous drainage requires the patient to have an external drain implanted for an extended period of time. This may lead to development of pancreatico-cutaneous fistulas; especially in patients with ductal disruption. In contrast to percutaneous drainage, an endoscopic approach allows placement of multiple drainage modalities through a single puncture site.

EUS permits a physician to visualize the entire abdominal cavity, assess the maturity of the wall, measure the distance between the collection and the luminal wall, and identify intervening vessels (collaterals) along the puncture site (Figures 2 and 3). Furthermore, the rates of technical success achieved when using EUS-guided drainage have been higher

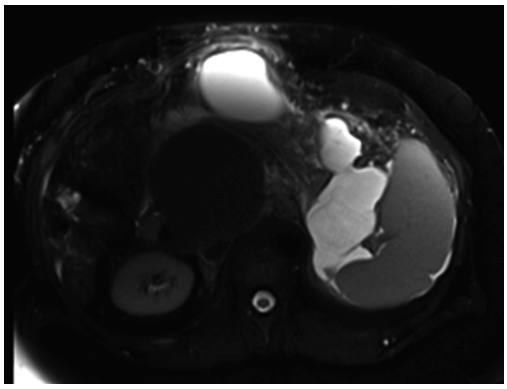

Figure 2 Selected magnetic resonance imaging frame showing a large peri-pancreatic pseudocyst extending from the pancreatic tail to the anterior abdominal wall in a patient with pancreatitis and splenic vein thrombosis.

than those achieved using conventional transmural endoscopic drainage techniques performed without EUS guidance[9]. EUS-guided drainage is the preferred modality in cases where there is no visible luminal bulge, portal hypertension and collateral formation are suspected, or when treating patients with coagulopathy[4,6,7].

SHOULD ERCP WITH TRANS-PAPILLARY DRAINAGE BE PERFORMED ON THESE PATIENTS?

Endoscopic drainage of PFCs may be performed either during endoscopic retrograde cholangiopancreatography (ERCP) with drainage through the main ampulla of Vater or *via* a transmural route - either the duodenum or stomach. Currently, no comparative or randomized studies have been reported from which solid data can be extracted regarding the preferred method for drainage. Only case series in which inconsistent methods and guidelines were used based on expert opinions have been published[10,11]. Based on the available published information, transpapillary drainage is preferred to EUS-guided transmural drainage as a first-step procedure for treating small fluid collections which communicate with the main pancreatic duct in the head or body of the pancreas (Figure 4). Moreover, most published cases which describe the use of EUS-guided drainage, fail to mention whether the patients had undergone ERCP prior to EUS-guided drainage. In one series of 116 patients, 15 patients received transpapillary drainage, 60 received transmural drainage, and 41 received both types of drainage. In that series, successful drainage was achieved in 88% of the patients[12]. However, there was no difference in the rates of success achieved using the different methods. Hence, little evidence exists to support a recommendation that pancreatic fluid collections should preferably be drained *via* the pancreatic papilla and pancreatic duct.

DOES LOCATION OF THE FLUID COLLECTION MATTER?

Transmural drainage has been attempted as a method for treating pancreatic pseudocysts (Figure 5A) and WON (Figure 5B) of suitable size, and located in the head, body or tail of the pancreas. However, this method requires that the distance between the lumen wall and cyst is < 1 cm. Due to their location in the lesser sac or extension to the pararenal space, PFCs in the pancreatic tail do not cause luminal compression, and can be accessed only by EUS. Varadarajulu *et al*[13] noted that the location of a pseudocyst is not predictive of treatment success. However, two cases of perforation have been reported when transgastric drainage was attempted for pseudocysts located in the uncinate process of the pancreas. This complication did not occur when uncinate pseudocysts were drained *via* the duodenum. Following transmural stenting, a low hanging pseudocyst in the uncinate region becomes decompressed, and may disconnect from the stomach wall, leading to perforation[14].

DOES THE AMOUNT OF INTERNAL DEBRIS MATTER?

In the context of draining PFCs, technical success refers to achieving access to a PFC and the placement of transmural stents, whereas clinical success means resolution of the collection. Very high clinical success rates (90%-100%) have been achieved when draining pseudocysts[9,12,15]. However, when treating cases of walled off pancreatic necrosis, the clinical success rates are generally poor. In a recent study of 211 patients with symptomatic PFCs, the reported success rate for treating sterile and infective pseudocysts was 93.5%, but only 63.2% when treating a WON[13]. Baron *et al*[16] reported a 92% success rate when performing pseudocyst drainage in patients without necrosis, compared to 72% in patients with necrosis. Although that study utilized non-EUS-guided endoscopic drainage, it illustrates the principle that outcomes achieved when performing endoscopic drainage of pseudocysts are superior to those achieved when draining collections with infected necrosis. In another study, drainage of a necrosis was clinically successful in only 25% of cases, but technically successful in 50% of cases[12]. If an aggressive endoscopic approach using endoscopic necrosectomy is adopted, success rates up to 81% can be achieved when treating a WON[17]; however, adjunctive surgical and percutaneous drainage may be required. Varadarajulu *et al*[18] suggested multiple transluminal gateway treatment (MTGT) for a WON, by which they attained a successful response in 92% of patients. In those cases, two or three transmural tracts were created between the necrotic cavity and gastrointestinal lumen

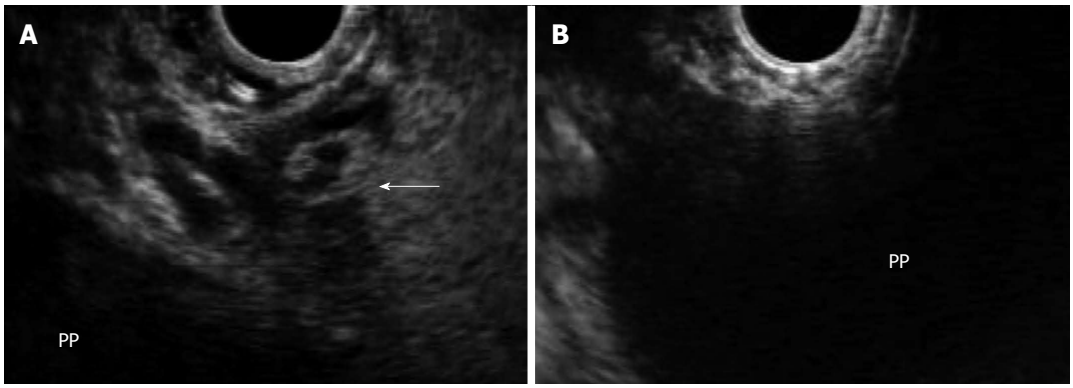

Figure 3 In the same patient, endoscopic ultrasound permits visualization of the pancreatic pseudocyst, assessment of wall maturity, determination of distance between the collection and the luminal wall, identification of intervening vessels or collaterals (arrow) (A), and selection of the optimal puncture site (B). PP: Pancreatic pseudocyst.

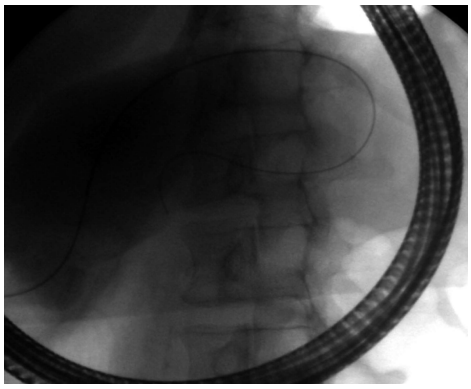

Figure 4 Fluoroscopic image showing transpapillary drainage of a pancreatic pseudocyst that is shared with the main pancreatic duct.

by using EUS guidance. While one tract was used to flush normal saline solution *via* a nasocystic catheter, multiple stents were deployed in the tracts to facilitate drainage of the necrotic contents.

As the amount of necrotic component in a collection increases, the success rate of draining the collection progressively decreases, unless aggressive endoscopic necrosectomy or concomitant percutaneous drainage is also used. The need for surgical intervention is also more common in these groups of patients. The revised Atlanta classification describes a WON as a well encapsulated fluid collection which occurs 4 wk in the setting of necrotic pancreatitis. However, this may not always be true, as previous necrotic collections can liquefy over time. A study conducted in India had patients undergo follow-up EUS examinations at 6 wk, 3 mo, and 6 mo after the onset of acute necrotic pancreatitis, and found that not all fluid collections following acute necrotic pancreatitis had a solid necrotic component. During the time period studied, the collections tended to decrease in size and their solid content tended to liquefy, with almost half of the PFCs being completely liquid at 6 mo[19].

Another study conducted by the same group examined 43 patients with a symptomatic WON treated by endoscopic drainage[20]. The WONs had a mean size of 9.95 ± 2.75 cm, and were found to contain < 10%, 10%-40%, and > 40% solid debris in 6, 33, and 4 patients, respectively. Patients with < 10% necrotic debris required only a single session of endoscopic drainage, whereas patients with 10%-40% solid debris required two or more sessions. Patients with > 40% solid debris required either direct endoscopic debridement or surgical necrosectomy. The extent of necrosis was significantly correlated with the type of treatment received by the patient ($r = 0.703$, $P < 0.001$).

CHOICE OF TRANSMURAL ACCESS DEVICES

The widespread use of EUS-guided PFC drainage has been limited by a lack of dedicated accessories. This factor necessitates using multiple steps to place a transluminal stent. The fluid collection is first visualized using a linear echoendoscope, and Doppler technology is used to ensure that no blood vessels lie in the line of puncture. The PFC is then visualized and punctured using a 19-gauge FNA needle, a cystotome or needle wire. After puncturing, a 0.035 guide wire is inserted into the PFC. When multiple stents need to be placed, some physicians prefer using a double guide wire approach in which two guide wires are simultaneously inserted after the first puncture[21]. A novel lumen-apposing self-expandable metal stent (AXIOS™ system, Xlumina; Mountain View, CA, United States) has recently been developed that can be deployed in a single step (Figures 6 and 7). The stent has a dumbbell-shaped configuration that foreshortens on deployment, and thereby minimizes the possibility of leakage or perforation[22].

CYSTOENTEROTOMY PATENCY DEVICES

A variety of stents have been used to maintain patency of the fistulous tract between the gut lumen and the

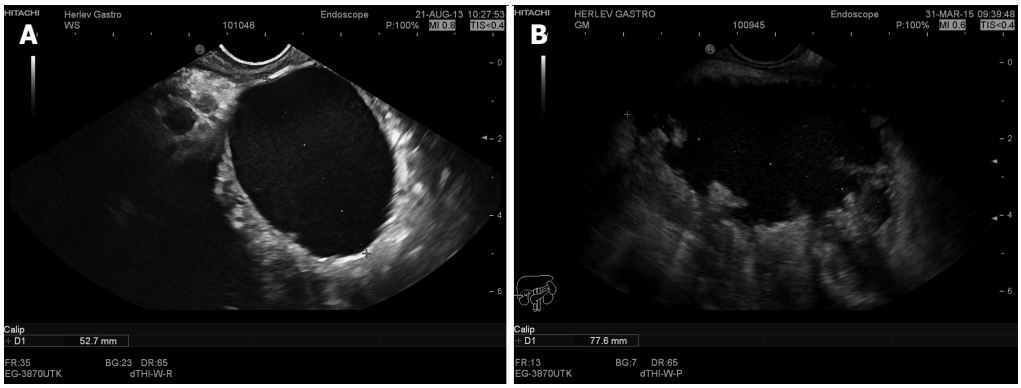

Figure 5 Endoscopic ultrasound image of a 5 cm chronic pseudocyst with a thin wall (A) or a 7.8 cm irregular pseudocyst with walled off necrosis (B).

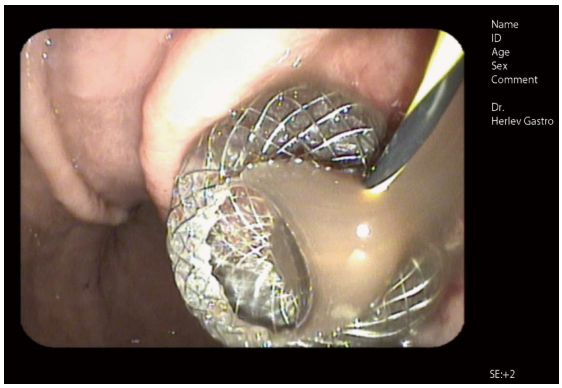

Figure 6 Endoscopic image of a self-expandable metal stent immediately after endoscopic ultrasound guided drainage (AXIOSTM system, Xlumina, Mountain View, CA, United States). Note the fluid floating through the stent opening. A guidewire extending through the stent lumen is still visible.

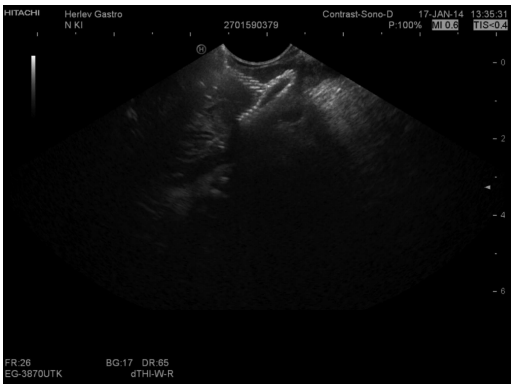

Figure 7 Corresponding endoscopic ultrasound image of a collapsed pancreatic pseudocyst immediately after drainage. Note reflexions from the stent mesh inside the collapsed cyst.

PFC. Single plastic stents (straight or double pigtail), multiple plastic stents, nasocystic drainage catheters, enteral metal stents, and biliary metal stents have all been tried. Some studies have also used combined modalities such as plastic stents in combination with nasocystic drainage catheters or double pigtail stents axially placed through a metal stent. While the available literature includes studies which used a variety of stents in combination, there is no clear evidence to suggest that metal stents are better than plastic stents, or that one type of plastic stent is better than another.

Pseudocysts

Lopes *et al*[23] used single plastic stents for draining pseudocysts. While drainage was successful in 93% of patients, 25% of patients experienced a recurrence. Those investigators also noted that complications occurred more frequently when using straight stents as compared to double pigtail stents; however, the difference was not statistically significant. Straight stents do not have anchorage, and thus can migrate more easily than double pigtail stents. Additionally, straight stents have been reported to cause bleeding and perforation.

Antillon *et al*[24] performed a single center prospective cohort study which examined the efficacy of single-step EUS-guided transmural drainage of pseudocysts. During the index procedure, complete resolution of the pseudocyst was achieved in 27 (82%) of 33 patients. Four additional patients (12%) had partial resolution (50% reduction in cyst size) accompanied by symptom resolution. Recurrence was observed in only one patient during a median follow-up period of 46 wk.

In a retrospective study, 87 consecutive patients with pancreatic pseudocysts were managed by EUS-guided drainage. Sixty-three patients with solid debris were drained *via* nasocystic drains placed alongside stents, while 24 patients with solid debris were drained *via* transmural stents. The short-term success rate among patients with viscous solid debris-laden fluid and whose pseudocysts were drained by both stents and nasocystic tubes was 3-fold greater than that among patients who were drained by stents alone (OR = 3.6; 95%CI: 1.2-10.7; P = 0.03). Long-term follow-up results showed a non-significant trend suggesting that pseudocysts were better resolved when debris was drained using nasocystic drains placed alongside stents compared to using stents alone (79% *vs* 58% respectively, OR = 2.7; P = 0.059). Moreover, when

draining debris-laden cysts, the rate of stent occlusion was higher when using stents alone rather than nasocystic drains placed alongside of stents (33% *vs* 13%, $P = 0.03$)[25].

Seewald *et al*[21] used a single step, simultaneous double-wire technique in conjunction with a prototype device to drain symptomatic cysts in eight patients. After puncturing a cyst, two 0.035-inch guide wires were simultaneously inserted into the cyst cavity. Next, transmural stenting was performed with an 8.5F double pigtail stent, and a 7 French nasocystic catheter was inserted into the cavity. The cavity was irrigated with a total of 1500 mL of saline solution daily (7-21 d duration) administered through the nasocystic catheter to prevent accumulation of pus and debris. Follow-up CT scans showed that all patients experienced complete resolution of their pseudocysts, and no recurrence was found during follow-up periods ranging 6 to 16 mo.

Due *et al*[26] described 10 patients who underwent pancreatic pseudocyst drainage; among whom, 7 patients received a 10 mm × 20 mm covered double-flanged metal stent. Three of the 7 patients who received the metal stent developed sepsis due to stent blockage, and one patient experienced persistent leakage. Two of the patients with stent blockage and the one patient with a leak ultimately required surgical intervention. Fabbri *et al*[27] reported the drainage of 20 patients with infected fluid collections using covered self-expanding metal stents (SEMS; 4 cm or 6 cm long, 10 mm diameter). The procedure was technically successful in all of the patients, and the treatment success rate was 90%. One month after insertion, the stents were removed, and the removal procedure was successful in all except one patient. Additionally, one stent had migrated and one patient required surgery. Several attempts have been made to provide better anchorage to SEMS by deploying plastic stents through them, and thereby reduce the likelihood of migration. Talreja *et al*[28] described a series of 16 patients with PFCs who underwent endoscopic drainage. SEMS (10 mm × 60 mm) were inserted, and double pigtail plastic stents were deployed through or alongside the metal stents to provide better anchorage. That study showed a 95% treatment response rate, and stent migration occurred in only one patient. Comparable results were reported by Penn *et al*[29], who used the same technique to drain pseudocysts.

A study from California[30] evaluated the safety and efficacy of EUS-guided drainage of PFCs using a one-step access device (NAVIX™, Xlumina), followed by placement of a fully covered SEMS. Eighteen patients with a PFC showing indeterminate adherence were enrolled. After 7-10 d, the fully covered SEMSs were removed and replaced with double-pigtail stents. When indicated, tract dilation and endoscopy-guided cyst debridement were performed. Fully covered SEMS placement was technically successful in all 18 patients, and there were no complications. Cyst resolution was achieved in 78% of the patients, and the median procedure time was 37.5 min. Berzosa *et al*[31] reported 100% technical and clinical success rates when using SEMSs, and found no instance of stent migration. The majority of SEMSs used in that study were tubular stents designed for transluminal drainage, including bile duct drainage. When used for transmural drainage, these SEMSs have some limitations, including a high risk of stent migration and a possibility of causing tissue injury and bleeding. As a result of those limitations, a novel large-diameter SEMS with bilateral flanges (the AXIOS stent) has been specifically designed for transmural drainage. It consists of a barbell-shaped, flexible, fully covered, self-expanding nitinol stent housed within a catheter-based delivery system. The new SEMS is available in two sizes (10 mm × 10 mm and 15 mm × 10 mm), and its 10 mm saddle length is designed to appose the stomach or duodenum to the PFC wall. In 2012, Itoi *et al*[32] first described the use of AXIOS stents in a series of 15 patients with symptomatic pseudocysts who underwent drainage. All stents were successfully deployed without complications; the pseudocysts resolved after a single drainage procedure, and the median time to removal was 35 d. Although one stent migrated into the stomach, the remaining 14 stents were found to be patent at the time of removal. Moreover, there was no pseudocyst recurrence during a median follow-up period of 11-mo. In 2013, a Spanish study[33] reported the use of AXIOS stents for pseudocyst drainage. In that study, the technical success rate was 88% (8/9 patients), as the stent delivery system failed in one case. However, no stent migration was reported and all stents were easily removed. Moreover, all patients experienced a complete resolution of their cyst.

Walled-off necrosis

A WON often leads to the severe clinical deterioration of a patient, and requires treatment with open debridement or endoscopic necrosectomy. Infection is a common complication which occurs during endoscopic drainage of pancreatic fluid collections, and is more common in patients with a WON than patients with a pseudocyst. This increased incidence of infection in cases of WON is presumably due to stent occlusion by solid debris and subsequent bacterial colonization. Hence, most physicians favor placing multiple stents, and especially in cases of WON. When performing MTGT, two or three transmural tracts are created between the necrotic cavity and gastrointestinal lumen under EUS guidance. While one tract is used to flush normal saline solution *via* a nasocystic catheter, multiple stents are inserted into the other tracts to facilitate drainage of necrotic contents. Varadarajulu *et al*[18] compared MTGT with conventional EUS-guided drainage in 60 patients with a symptomatic WON. Twelve of the patients were managed by MTGT and 48

by conventional drainage. Treatment was successful in 11 of 12 (91.7%) patients managed by MTGT *vs* 25 of 48 (52.1%) patients managed by conventional drainage (P = 0.01). Although 1 patient in the MTGT cohort required endoscopic necrosectomy, 17 patients who received conventional drainage required surgery, 3 underwent endoscopic necrosectomy, and 3 died of multiple-organ failure. A multicenter prospective study[22] from the United States evaluated the outcome of AXIOS stent placement in 33 patients with symptomatic pancreatic pseudocysts and WONs. The devices were successfully placed under endoscopic ultrasound guidance in 30 patients (91%), while the remaining 3 patients each received two double-pigtail stents. One subject could not be evaluated due to a pseudoaneurysm. Among the 29 patients who received an AXIOS stent, 27 patients (93%) showed resolution of their PFC, and stent migration was noted in only one patient. In a large multicenter trial involving 15 European centers, 61 patients with either a pseudocyst (n = 46, 75%) or WON (n = 15, 25%) were drained using AXIOS stents. In that study, stent placement was judged to be technically successful in 60 (98%) of the 61 patients. Clinical success, defined as resolution of clinical symptoms combined with a decrease in the PFC size to $\leqslant$ 2 cm on imaging, was achieved in 93% of patients with a pancreatic pseudocyst and 81% of patients with a WON. Treatment failure occurred in nine patients (16%), including four patients who required surgical intervention. Stent removal was performed in 82% of patients after a median time of 32 d, and the removal procedure was rated as "easy" by all but one patient. Endoscopic stent removal was not performed in a total of 10 patients due to stent migration (n = 3), stent dislodgement during necrosectomy (n = 3), stent removal during surgery (n = 2), or patient refusal (n = 2).

SINGLE OR MULTIPLE STENTS?

No randomized control trial has compared the benefits of using a single plastic stent *vs* multiple plastic stents *vs* a metal stent for treating a pseudocyst. However, retrospective studies have shown that insertion of even a single stent provides high rates of clinical resolution. This is probably because stent occlusion has not occurred due to the absence of solid necrotic debris. A recent meta-analysis of 14 studies involving 698 patients found no difference in the rates of treatment success between patients managed with multiple plastic stents *vs* metal stents (89%, 95%CI: 87-91 *vs* 87% 95%CI: 76 to 91; P = 0.22, respectively). Furthermore, the two cohorts showed no difference in their respective rates of adverse events or pseudocyst recurrence[34]. However, these results may not apply in cases of WON, because stent occlusion and consequent treatment failure are likely occurrences, and the chances of achieving clinical resolution and treatment success depend on providing adequate drainage. This notion is reinforced by the successful use of MTGT to treat cases of WON as proposed by Varadarajulu *et al*[18], who placed multiple stents at different sites in conjunction with a nasocystic drain to achieve resolution of a WON in > 90% of cases.

DEFINITION OF CLINICAL AND RADIOLOGICAL SUCCESS

Technical success is defined as the ability to access and drain a pancreatic pseudocyst by placement of a stent. Treatment success involves both clinical and radiologic improvement. Clinical success refers to the resolution of symptoms that prompted an intervention. Radiologic success refers to a decrease in the size of a cyst or its resolution. In a randomized clinical trial comparing endoscopic and surgical drainage of pseudocysts, treatment success for endoscopy was defined as complete resolution or a decrease in the size of a fluid collection to 2 cm or less as seen on a CT scan, in combination with the resolution of clinical symptoms as determined at an 8-wk outpatient follow-up evaluation[8]. Follow-up is usually done using upper endoscopies and imaging techniques (either a CT scan or abdominal ultrasound).

EFFICACY OF EUS-GUIDED DRAINAGE OF PANCREATIC FLUID COLLECTIONS

Very high clinical success rates (90%-100%) have been achieved by draining pseudocysts[9,12,23]; however, less data is available concerning the clinical success rates achieved when draining an abscess. While high treatment success rates ranging from 80%[35] to > 90%[12] have been reported, the rates for clinical resolution of a WON are generally poor. In a recent study of 211 patients with symptomatic PFCs, the rate of success in treating sterile and infected pseudocysts was 93.5%, compared to only 63.2% when treating WONs[36]. However, the success rate for treating WONs can be improved to 81%[17] if an aggressive endoscopic approach using endoscopic necrosectomy is adopted; although adjunctive surgical and percutaneous drainage may be needed. Varadarajulu *et al*[18] suggested multiple transluminal gateway treatment for a WON, and achieved a clinically positive response in 92% of patients when using this method. In a retrospective review of 31 patients who received EUS-guided drainage of fluid collections after pancreatic resection, EUS-guided drainage was performed with a technical success rate of 100%, and clinical success was achieved in 29 of the 31 patients (93%)[37].

COMPLICATIONS AND RISKS OF EUS-GUIDED TRANSMURAL DRAINAGE

When using EUS-guided transmural drainage, the rates

of complication range from 1%-18%[12,24,36,38-40], and complications most frequently manifest as bleeding, perforation, secondary infection or stent migration. A retrospective study conducted by Varadarajulu *et al*[13] reported a significantly higher complication frequency in cases of pancreatic necrosis (15%) when compared to cases involving a pseudocyst or abscess (5%). Secondary infection is caused by contamination of an incompletely drained WON or pseudocyst resulting from premature stent occlusion or its uneven collapse, and occurs in about 10% of cases. The perforation risk increases when a pseudocyst wall is poorly defined or is located > 1 cm from the intestinal lumen. Very few cases of procedure-related mortality have been reported, and the ones that have were mainly related to bleeding[12,41,42]. Because surgery is required in 5%-11% of cases, most complications are conservatively managed by an interventional radiologist or endoscopy[43]. Complications such as pneumothorax, air embolism, and intra-abdominal abscess have been seldom reported in the literature.

WHEN TO CONSIDER SURGERY

Walled-off pancreatic collections can be surgically managed with either an open surgical procedure or a laparoscopic approach. Most surgical literature has described the use of open surgical drainage procedures. Open surgical drainage can be accomplished *via* cystgastrostomy, cystenterostomy (direct drainage or *via* a Roux limb) or resection. However, drainage can also be accomplished using laparoscopic techniques. Laparoscopic cystgastrostomy can be performed *via* an anterior transgastric approach or a posterior approach through the lesser sac. The latter approach requires only a single gastrotomy in continuity with the walled-off pancreatic fluid collection. During the last 10 years, endoscopic drainage has come to the forefront and demonstrated efficacy comparable to that of surgical drainage; additionally, it is less expensive to perform and requires a shorter hospital stay.

Surgical drainage is a multidisciplinary decision and should only be considered for patients who have experienced previous endoscopic failures, disease recurrence following a successful endoscopic drainage, and patients who do not satisfy the criteria for endoscopic or percutaneous drainage. In 2011, Seewald *et al*[44] published a paper describing the long term results of patients who underwent endoscopic drainage of PFCs. Their retrospective analysis of 80 patients with symptomatic PFCs showed that fluid collections were clinically resolved by endoscopic methods in 67 (83.8%) patients, and surgery was required for 13 patients (perforation: four patients; endoscopically inaccessible areas: two patients; inadequate drainage: seven patients). Five patients required surgery within 6 mo after their first treatment due to recurrent fluid collection. Moreover, during a mean followup period of 31 mo, an additional four patients required surgery due to recurrent collections as a consequence of underlying pancreatic duct abnormalities that could not be treated endoscopically. The long-term success rate of endoscopic treatment was 72.5% (58/80 patients), and 28% of patients required surgery.

The traditional approach to treating necrotizing pancreatitis accompanied by a secondary infection of necrotic tissue is open necrosectomy to completely remove the infected necrotic tissue. However, this invasive approach is associated with high rates of complications and death (11%-39%), as well as a risk of long-term pancreatic insufficiency. The Dutch pancreatitis study group showed that an incremental approach consisting of percutaneous drainage followed, if necessary, by minimally invasive retroperitoneal necrosectomy is a better strategy than open necrosectomy for treating patients with necrotizing pancreatitis and a secondary infection[45]. Additionally, new-onset multiple-organ failure occurred less often in patients treated using the incremental approach than in those treated *via* open necrosectomy (12% *vs* 40%, $P = 0.002$).

FUTURE DIRECTIONS

The revised Atlanta classification of acute pancreatitis better defines local complications which can be associated with pancreatitis: acute PFC, acute necrotic collection, pseudocyst formation, and WON. In recent decades, interventional EUS has been increasingly utilized in the management of these local complications. With development of improved and novel endoscopic devices dedicated to transmural drainage of fluid and necrotic debris (access and patency devices), we believe that EUS will become an indispensable part of procedures used to diagnose PFCs and image guided interventions.

The evidence provided in this review suggests that the amount of necrosis is the most important predictor of a successful outcome following drainage of a PFC. Hence, it seems logical to classify these collections based on their percentage of necrotic component or debris as indicated by radiological imaging or EUS. Thus we propose using a classification system in which fluid collections can be categorized into 3 groups: those with a solid component < 20%, those with a solid component between 20%-50%, and those with solid a component > 50%. As patient clinical outcomes are directly related to the type of fluid collection being treated, it is important to accurately distinguish a PFC before initiating intervention. In this review, the authors proposed using a management algorithm based on the amount of internal debris present in a PFC (Figure 8). For PFCs with < 20% internal debris, transmural drainage with 1-2 double pigtail plastic stents or a lumen apposing metal stent would probably be sufficient. For PFCs with > 50% internal debris, endoscopic necrosectomy with placement multiple

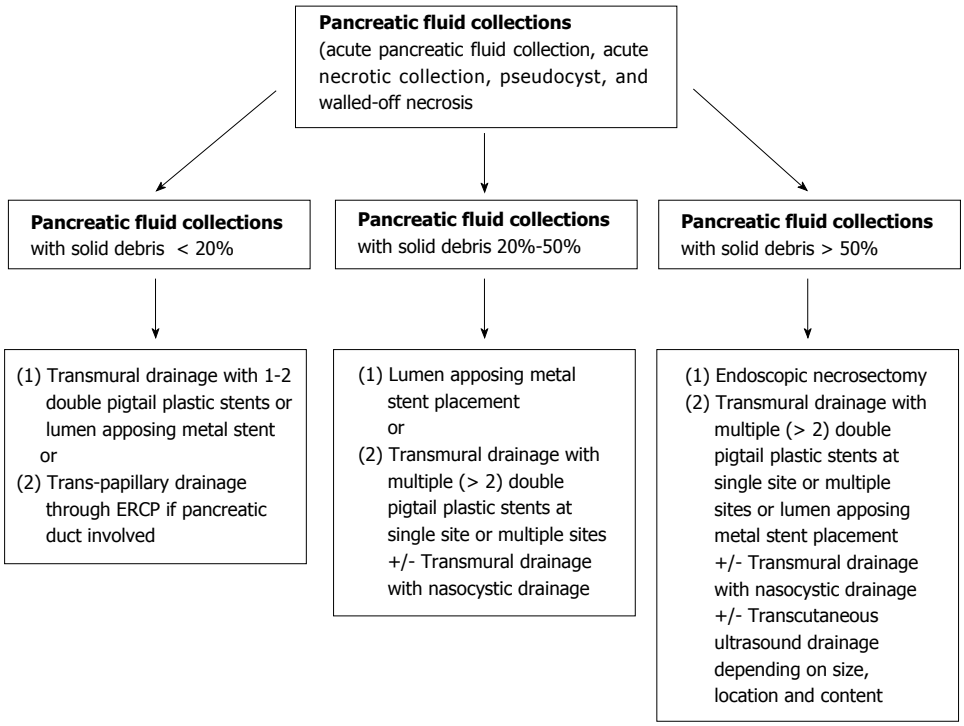

Figure 8 Authors' proposed endoscopic ultrasound-guided management algorithm based on the amount of internal debris inside a pancreatic fluid collection.

plastic stents or a lumen apposing metal is required. If the pancreatic duct is connected to the cyst, trans-papillary drainage *via* ERCP can be performed on any of these patients at the discretion of an endoscopist.

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SYSTEMATIC REVIEWS

Animal models of human colorectal cancer: Current status, uses and limitations

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Abstract

AIM: To make orthotopic colon cancer murine models a more clearly understood subject. The orthotopic tumor models have been found to be more relevant in replicating the human disease process as compared to heterotopic models, many techniques for making orthotopic colorectal murine models have been reported.

METHODS: We evaluated the current literature for various reported orthotopic colon cancer models to understand their techniques, advantages and limitations. An extensive literature review was performed by searching the National Library of Medicine Database (PubMed) using MeSH terms animal model; colon cancer; orthotopic model; murine model. Twenty studies related to colon cancer orthotopic xenograft model were evaluated in detail and discussed here.

RESULTS: The detailed analysis of all relevant reports on orthotopic model showed tumor take rate between 42%-100%. While models using the enema technique and minimally invasive technique have reported development of tumor from mucosa with tumor take rate between 87%-100% with metastasis in 76%-90%.

CONCLUSION: Over the years, the increased understanding of the murine models of human colon cancer has resulted in the development of various models. Each reported model has some limitations. These latest models have opened up new doors for continuing cancer research for not only understanding the colon cancer pathogenesis but also aid in the development of newer chemotherapeutic drugs as they mimic the human disease closely.

Key words: Murine model; Colon cancer; Colon cancer murine model; Orthotopic model; Animal model; Colon cancer animal model; Colorectal cancer; Cancer model; Colorectal cancer; Colorectal cancer animal model

Core tip: The murine models of colon cancer represent an important tool for understanding the etiopathogenesis and evaluating management strategies for colorectal cancer, thus representing a resource of immense potential in cancer research. Over the years, the increased understanding of the murine models of human colon cancer have resulted in the development of various models. We evaluated the current literature for various reported orthotopic colon cancer models. Our paper discusses the techniques, results, advantages and limitations of the presently available murine models of colorectal cancer so that a researcher can choose an appropriate colorectal cancer murine model which fits their research goals.

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INTRODUCTION

National Cancer Institute and SEER (Surveillance, Epidemiology and End Result) Program 2011, reported the incidence of colon cancer as 43.3 and the number of people dying from colon cancer as 15.9 per 100000 men and women per year. Colon cancer remains the third most common cancer among men as well as women and 2nd leading cause of cancer related deaths in United States[1].

Recently, there have been many advances in the understanding of colon cancer epidemiology, pathogenesis, pathology, chemoprevention and therapeutic options, which seem to have emanated from continuing basic and clinical research. Host genetic factors play a critical role in the patho-physiology of most human cancers. The immense biological complexity of colon cancer has kindled the development of more apt research design that could simulate in a natural and spontaneous fashion the pathophysiologic features of cancer biology. Rodent models have many desired attributes and they share a wide variety of characteristics with human that has proven to be important in comprehending many complex molecular facets of colon cancer[2]. They also act as an invaluable tool in the development of newer chemotherapeutic drugs. This has made the laboratory mouse (Mus musculus) as one of the most attractive entity in oncologic research[3,4].

Thus there is a constant endeavor to develop an animal model that closely mimics the malignant disease process of humans. Although many animal models were tried but as per NCI data only two animal models based on breast and colon cancer histology are successfully used in preclinical trials[5,6]. Together with that further studies have displayed that the human xenograft models have shown better results than the murine allograft models in drug development. Till date more than 100000 rodents have been sacrificed for the development of chemotherapeutic drugs. Along with that, this fact also remains that, millions of people all over the world are alive today because of the animal research[7-9].

Colorectal cancer basic research has grown itself on the animal models, which have now become the pillars for understanding the pathogenesis and for developing newer chemotherapeutic drugs. The murine models depict a resource of immense potential, as an intricate disorder like colon cancer can be simultaneously witnessed and manipulated by the researchers. Our knowledge in this field has evolved a lot, and many mouse models have been reported, but each has certain limitations as there is no spontaneous colon cancer and a carcinogen is required for tumor induction in the rodents. Also, many of the mouse models have inter-animal variability in the development of tumors in the intestine. In spite of that animal models have become an important tool in better understanding the effect of genetic alterations on the disease process[10-12].

We reviewed the literature for evaluating the different techniques of developing orthotopic xenograft murine colorectal cancer models, their advantages and limitations. This was done to offer the investigators an appropriate model for research in order to obtain robust and translatable data to aid further understanding of colorectal cancer.

MATERIALS AND METHODS

An extensive review of the literature on murine models of colon carcinogenesis was performed by searching the National Library of Medicine Database (PubMed) on 12/28/2014. The MEDLINE search was made using MeSH terms: animal model; chemoprevention; colon-carcinogenesis; min-mice; colon cancer; colorectal cancer; xenograft, heterotopic model, orthotopic model, murine model. We found a total of 5622 literatures by searching above key words. All the 477 relevant articles were analyzed and interpreted in detail and of these 20 manuscripts pertaining to colon cancer orthotopic xenograft model were included in this review.

RESULTS

Based on detailed analysis of all relevant reports on orthotopic colorectal cancer xenograft model, we categorized them into different groups according to the techniques used to create these models. The outcomes using different techniques in various studies are discussed in Table 1.

Open surgical technique

Ogata *et al*[13] in 1998, used *in vivo* KM12 SM cell lines

Table 1 Characteristics of reported orthotopic murine models of human colon cancer

Technique	Author	Year	Mice type	Cell-line used	Tumor development (%)/origin	Lymph node metastasis (%)	Distant metastasis (%)
Open surgical	Ogata	1998	Nude	Human colon carcinoma KM12 SM cells	100/Mural	25	10 (Lung) 50 (Liver)
	Hackl	2012	Nude	Human colon cancer cells, transfected with hCG and luciferase	87.5-100/Mural	50	25 (Lung) 50 (Liver)
	Priolli	2012	Nude	Colorectal adenocarcinoma cell line (CCL-218)	42.8/Mural	00	00
Enema model	Takahashi	2004	Nude	Human colon cancer cells, LS174T	95/Mucosal	NS	NS
	Kishimoto	2013	Nude	Mouse rectal cancer cells, expressing green fluorescent protein (GFP)	100/Mucosal	90	90 (Lung) 09 (Liver)
Microinjection	Donigan	2009	Nude	Murine colon cancer (CT26) cells	65/Mural	NA	3.3
	Zigmond	2011	Nude	Murine colon cancer C57BL/6 CRC cells Human colon cancer cells SW620, SW480 and LS174T	95/Mural	NA	NA
Transanal low dose electrocoagulation	Bhullar	2010	Nude/ SCID	Murine cell line CRL-2639, CRL-2638 Human cell line HT-29, LS-174T	92-100/Mucosal 58-100/Mucosal	50-100 33-83	41-50 0-83.33

and injected them into cecal wall of nude mice through an open surgical technique for making the orthotopic xenograft model. They reported tumor take rate of 100% and metastasis to the regional mesenteric lymph nodes in 25% and to liver in 50%.

Hackl *et al*[14] in 2012, injected human colon cancer cell lines HT 29 and HCT 116 transfected with human chorionic gonadotropin (b-hCG) and luciferase, orthotopically into the caecal wall of severe combined immunodeficient (SCID) mice. The developing tumor produces b-hCG and luminescent protein luciferin. The levels of these markers correlate with tumor burden, completeness of resection and recurrence. They reported tumour take rates between 87% to 100%, metastases to lymph nodes and liver in 50% and lungs in 25% following intracaecal cell injection.

Priolli *et al*[15] in 2012, also used the open surgical method for colonic diversion with distal fistula formation. This was followed by injecting of WiDR colorectal (CCL-218) adenocarcinoma cell line into the submucosa of the fistula made in the mice. Tumor growth was reported in 42.8% mice with metastasis in none.

Enema technique model

Takahashi *et al*[16] in 2004, developed a technique by inducing short term colitis in nude mice by an irritant agent, 3% dextran sulfate sodium (DSS) followed by instillation of human colon cancer cells LS174T transanally. They reported a tumor take rate of 95% in rectum after 2 wk but could not observe any significant metastasis.

Kishimoto *et al*[17] in 2013, used 4% acetic acid solution for two minutes, followed by flushing with 6 ml phosphate buffered saline (PBS) in order to disrupt the epithelial cell layer of the distal rectal mucosa followed by mouse colorectal cancer cell line CT-26 and the human colorectal cancer cell line HCT-116 cells, expressing green fluorescent protein (GFP) were instilled transanally. Authors noted rectal tumor development in 100% of the mice. Spontaneous lymph node metastasis and lung metastasis were found in over 90% of mice.

Microinjection technique

Donigan *et al*[18] in 2009, used an optical microscope, to inject murine colon cancer (CT-26) cells into the rectal wall of the nude mice under magnification (10-100 ×) with an overall tumor take rate of 65% and distant metastasis in 3.3%.

Using a similar technique, Zigmond *et al*[19] in 2011 reported a murine model in which the murine and human colon cancer tumor cells - C57BL/6 CRC tumor cells and SW620, SW480 and LS174T respectively were injected into the wall of distal rectum through a murine colonoscope (Coloview- Karl Storz). They reported tumor take rate of 95% with no metastasis.

Transanal low dose electrocoagulation technique

Bhullar *et al*[20] in 2010, transanally instilled human (LS-174T and HT-29) and murine (CRL-2638 and CRL-2639) colon cancer cell lines in SCID and nude mice, after transanal low dose mucosal electrocoagulation of the colon. Overall tumors developed in 87.5% of mice (42/48) *i.e.,* 12 of 12 and 11 of 12 mice with murine tumor lines (CRL-2638 and CRL-2639, respectively) and in 7 of 12 and 12 of 12 mice with human tumor lines (HT-29 and LS-174T, respectively). While overall lymph nodal and distant metastasis was found in 66.66% cases (32/48) *i.e.,* 12 of 12 and 6 of 12 mice with murine tumor lines (CRL-2638 and CRL-2639, respectively) and in 10 of 12 and 4 of 12 mice with human tumor lines (HT-29 and LS-174T, respectively).

DISCUSSION

After reviewing all the current literature related to orthotopic xenograft murine colorectal cancer models

Figure 1 Showing a heterotropic colonic tumor on the flank of the mouse. (Image used from author's personal collection).

in detail, it is clear that there has been enormous development in the murine models over time. The latest models are more applicative with high tumor take rates, but still they have some limitations.

The historical murine cancer models were xenograft heterotopic models which laid the foundation for the development of newer orthotopic models. These xenograft heterotopic models were traditionally made by subcutaneous implantation of human colon cancer cells into either, nude T-cell deficient mice or NOD SCID mice (non-obese diabetic/severe combined immunodeficiency). The formed tumor is located externally in this model, so, its growth can be easily and accurately monitored (Figure 1).

Though making a heterotopic model is easy, but it has multiple pitfalls. It does not mimic the human disease process because of the extra - anatomical location of the tumor and the absence of metastasis from the subcutaneous location made the model inappropriate for study of the spontaneous metastatic process. In the present scenario, the only role of the heterotopic model is its use in making the orthotopic model. Subcutaneously grown tumor is resected after euthanizing the animal and small parts of the tumor are then embedded on the colon to make the orthotopic model[21,22]. The technically more advanced orthotopic xenograft murine models are formed by implanting colorectal cancer cells into colon and they are superior to the traditional subcutaneous xenograft tumor models. The orthotopic model resembles the entire spectrum of colorectal cancer ranging from in situ tumor to metastatic tumor. Commercially available cell lines can be used for making the orthotopic tumors, but these cell lines are altered by years of cultures in vitro, so injecting cells directly from tumors is preferred[23-25].

As discussed earlier, there are various techniques for instilling cancer cells into colon or rectum of murine animal models.

To begin with, investigators have used open surgery methods of implanting tumor cells with many variations to study colorectal tumorigenesis in animal models. Pocard *et al*[26] in 1996 made subcutaneous xenografts by injection of cancer cells subcutaneously in flanks of the mice. When the tumors acquired a size of > 1 cm^3, the mice were euthanized and the tumor sliced into pieces measuring 2 mm × 2 mm × 2 mm. These sections were later implanted under anesthesia through a midline abdominal incision to the serosal surface of cecum and fixed with a stitch and abdomen closed. Identically, in another model, the tumor cells were injected into colonic submucosa from the cecal serosa rather than fixing with a stitch.

Ogata *et al*[13] in 1998, followed the similar technique for orthotopic inoculation of human colonic cancer cell lines KM12 SM into the cecal wall of nude mice. They found tumors growing in 100% mice and metastasis to the regional mesenteric lymph nodes in 25% and to liver in 50%[27-29].

The open orthotopic models have multiple downsides. The initial immune response following the open and minimally invasive colon resections checks its authenticity. They do not cause mesenteric and retroperitoneal lymphatic metastases. Furthermore, the prior proliferation necessary in these models alters the growth and dissemination potential of the cell lines[30-33].

In 2012, Hackl *et al*[14] presented only study of its kind which thoroughly compares all three ways of human xenograft models of CRC *i.e.,* subcutaneous, orthotopic and intrasplenic. In this review we have discussed the technical aspects of only orthotopic xenograft done by open method. This new technique to execute xenografts from human colon cancer cell lines, transfected with luciferase and beta-hCG. In the Orthotopic model, open surgical technique was used. Cecum was assessed through midline incision following which trypan blue was injected into cecal wall. The cecum was then being stabilized carefully on a scalpel holder to prevent spillage of tumor cells. Using a 10 mL Hamilton syringe and 30G needle 5 ml cells (5×10^5) were infused into the cecal wall under 4 × magnification. Though this approach has a high tumor take rate of 87.5%-100% but the pitfall is that it is an intricated technique requiring specialized training, expertise hands and suitable instruments. Intrasplenic tumor cell injection was followed by a speedy colonization of the cancer cells to liver and lungs, within a day, which could not be explained by spontaneous metastasis. Mice yield to considerable tumor load within a few weeks and drug efficacy also could not be studied.

Priolli *et al*[15] in 2012 designed another open surgical method in which colon diversion and distal fistula formed, cancer cells were infused in the submucosa of the fistula. Scintigraphy with 99mTc-MIBI was performed to identify the tumor and monitor its growth. Tumors developed spontaneously in the

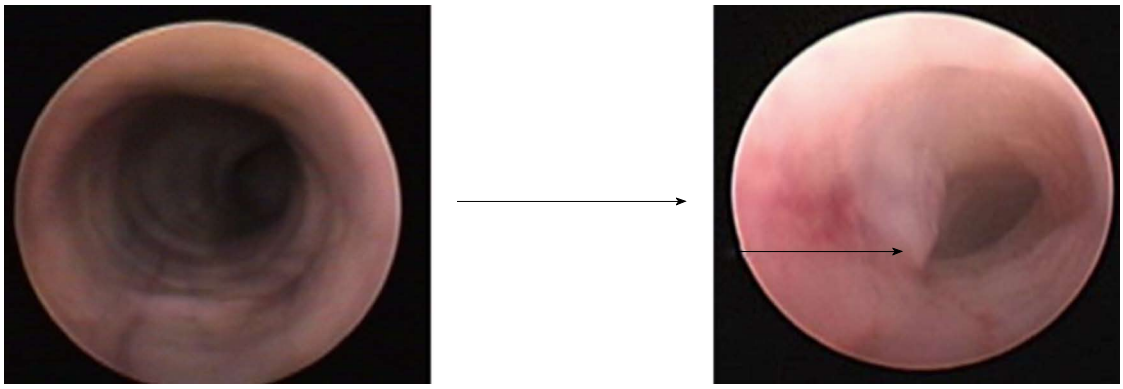

Figure 2 Colonoscopic picture on the left show a normal murine colon with smooth circumferential mucosa. While the colonoscopic picture on the right shows the orthotopic tumor growth (marked). (Image used from author's personal collection).

submucosa and histological examination revealed poorly differentiated tumor cells identically to the cells implanted. Distal fistula allowed easy monitoring of the tumor growth and assessing the effect of cytotoxic therapy. Tumor metastasis was not seen which may be due to insufficient time for tumor spread, insufficient number of cells inoculated or genotypic variance of tumor cells regarding invasiveness and metastatic potential.

Next is the enema model technique which requires induction of colitis followed by instillation of cancer cells transanally. Takahashi and associates in 2004 used a distinct non-surgical technique of orthotopic implantation. They infused 3% dextran sulfate sodium (DSS) to induce colitis followed by which cancer cells were instilled transanally. Clapper *et al*[34] also analyzed this approach by cyclically administering DSS, which generated colorectal dysplasia and carcinoma with similar pathological characteristics as humans. The frequency and profusion of these lesions differ, depending on the genre of mouse used, dose and schedule of DSS. Also the advancement of these tumors to invasive cancer could be potentiated by delivering DSS simultaneously with a known colon carcinogen {azoxymethane (AOM), 2- amino-3-methylimidazo[4,5-fl] quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)} or iron[16].

Kishimoto *et al*[17] in 2013 also used similar technique for orthotopic implantation of mouse rectal cancer cells, stably expressing green fluorescent protein (GFP). Instilling acetic acid solution dis-integrated the epithelial cell layer of the rectal mucosa. CT26-GFP cells in Matrigel were injected in rectal mucosa 4mm from rectal ring. The anus was promptly sealed by tape to restrain cell leakage. The inoculated tumor cells were noninvasively observed by fluorescence microscope. The mice were then euthanized for histological studies and to investigate potential metastasis. The carcinoma developed in 100% of mice, it initiated in rectal mucosa and then invaded submucosa. The tumor metastasized to the lymph nodes and lungs in 90% while dissemination to the liver was seen in only 9% of mice, contrary to the fact that liver is the most common site for metastasis of colorectal carcinoma. The authors elucidated it by the development of tumor in the lower rectum, which primarily drains in systemic venous circulation. As tumors arise only in rectum, which may not be useful in colonic studies, is major limitation of this model.

Another developed modality was microinjection technique which involves instillation of tumor cells directly into the colonic wall by microinjection. Donigan *et al*[18] in 2009 has devised this non-operative technique by using optical microscope in which the colon cancer cells were injected into the rectal wall of the mouse under magnification (10-100 ×) with an overall uptake rate of 65%. But the hitch was that the injection of the cells into the rectal wall evolved in cancers not emanating from the mucosa. Using the identical notion, Zigmond *et al*[19] in 2011 reported a murine model in which the tumor cells were injected into the wall of colon through a murine colonoscope (Coloview-Karl Storz). They reported tumor take rate of 95% with no metastasis. But, by the use of coloview the tumor cells can be implantated only in distal rectum[35,36].

For overcoming problems and deficiencies with the previously described colorectal cancer murine models, Bhullar *et al*[20] 2010 came up with a very impressive noninvasive technique *i.e.,* transanal low dose electrocoagulation technique. They reported a true orthotopic murine model using tumor cell implantation after low-dose colonic mucosal coagulation through a small transanal electrode resulting in limited mucosal injury. Subsequently, they instilled the tumor cells transanally on the injured mucosa, which then proliferated in the non-ischemic bed. The resultant tumors grew from the mucosal surface and subsequently involved the deeper colonic layers. The colonoscopic (coloview) view of the normal mouse colon shows a smooth circumferential mucosa, while the orthotopically grown tumor from the mucosal surface can be easily detected and followed up (Figure 2).

Tumors developed in 87.5% of the mice (42/48)

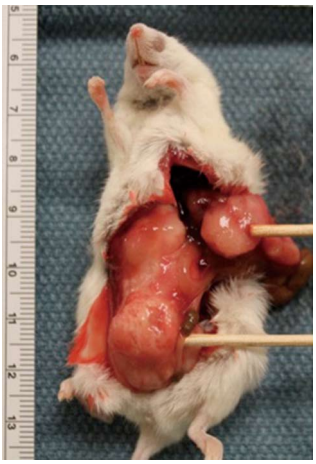

Figure 3 Showing the large orthotopic colon cancer tumor involving the surrounding structures (Lower marker). Also shown is the metastatic matted group of lymph nodes (Upper marker). (Image used from author's personal collection).

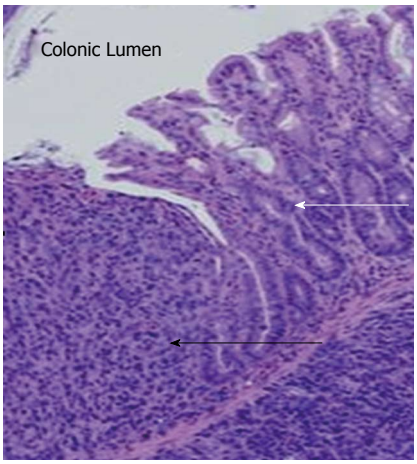

Figure 4 Showing the orthotopic colon tumor growing from the colonic mucosal surface (Black marker) (hematoxylin-eosin staining, magnification × 100). There is surrounding normal colonic mucosa (white marker). Adapted from Ref. [20].

i.e., 12 of 12 and 11 of 12 mice with murine tumor lines (CRL-2638 and CRL 2639, respectively) and in 7 of 12 and 12 of 12 mice with human tumor lines (HT-29 and LS 174T, respectively). The tumors were initially smaller, but over time grew bigger to invade the surrounding structures like the bladder, pelvic bones, *etc*. (Figure 3). Histologic evaluation revealed that these tumors grew from the mucosal lining (Figure 4). Overall Metastatic disease *i.e.,* lymph nodal in 32/48 *i.e.,* 66.66%, while liver, omentum and peritoneum involvement was seen in 43.75% (21/48) of cases. Orthotopic model using HT-29, LS 174T human cell lines showed tumor development in 58%-100% cases, lymph node and liver metastasis seen in 33-83.33% cases[20].

This noninvasive model has succeeded in dealing with most of the limitations of the antecedent models as: (1) there is no need of laparotomy for tumor implantation at colon or rectum; (2) easy to learn noninvasive technique with high reproducibility; (3) it closely mimics the natural development of disease process in humans; and (4) tumor uptake was 100% and metastasis was 83.33% with human cell line (LS 174T).

This model has come as a boon in the research for human colon cancer. It has enormous potentials and can be used to study tumor development, metastasis, and evaluation of novel chemotherapeutics for the colorectal cancer.

With the escalating worldliness of modelling carcinogenesis in mice, an intricate disorder can be simultaneously witnessed and manipulated by the researchers. The recently developed orthotopic xenograft murine models of colon carcinoma have added a new dimension in the research of colorectal cancer by overcoming limitations of the earlier ones.

The model by our group has overcome most of the shortcomings of the antecedent models and has opened new way for understanding disease process and testing therapeutic drugs that can potentially benefit patients of human colorectal cancer.

At the same time better understanding of the disease process along with the improved technology available to the future researchers, we can expect more advancement in these orthotopic murine models.

COMMENTS

Background

The murine models of colon cancer represent an important tool for understanding the etiopathogenesis and evaluating management strategies for colorectal cancer, thus representing a resource of immense potential in cancer medicine. An increasing knowledge in this field has led to development of various murine models, for human colon cancer but they have many limitations.

Research frontiers

The present model has enormous potentials and it will open up new dimensions of knowledge in study biology and evaluation of novel chemotherapeutics for the colorectal cancer.

Innovations and breakthroughs

This is easy to learn and highly reproducible noninvasive technique that closely mimics the natural disease process of carcinoma colon in humans with tumor uptake and metastasis of 100% and 83.33% respectively.

Applications

This will help in better understanding of disease process and testing therapeutic drugs that can potentially benefit patients of human colorectal cancer.

Peer-review

The authors reported the development of various orthotopic murine model of colorectal cancer and well summarized the advantages and disadvantages of each method with excellent review.

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SYSTEMATIC REVIEWS

Novel understanding of ABC transporters ABCB1/MDR/P-glycoprotein, ABCC2/MRP2, and ABCG2/BCRP in colorectal pathophysiology

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Abstract

AIM: To evaluate ATP-binding cassette (ABC) transporters in colonic pathophysiology as they had recently been related to colorectal cancer (CRC) development.

METHODS: Literature search was conducted on PubMed using combinations of the following terms: ABC transporters, ATP binding cassette transporter proteins, inflammatory bowel disease, ulcerative, colitis, Crohns disease, colorectal cancer, colitis, intestinal inflammation, intestinal carcinogenesis, ABCB1/P-glycoprotein (P-gp/CD243/MDR1), ABCC2/multidrug resistance protein 2 (MRP2) and ABCG2/breast cancer resistance protein (BCRP), *Abcb1/Mdr1a*, *abcc2/Mrp2*, *abcg2/Bcrp*, knock-out mice, tight junction, membrane lipid function.

RESULTS: Recently, human studies reported that

changes in the levels of ABC transporters were early events in the adenoma-carcinoma sequence leading to CRC. A link between ABCB1, high fat diet and gut microbes in relation to colitis was suggested by the animal studies. The finding that colitis was preceded by altered gut bacterial composition suggests that deletion of *Abcb1* leads to fundamental changes of host-microbiota interaction. Also, high fat diet increases the frequency and severity of colitis in specific pathogen-free *Abcb1* KO mice. The *Abcb1* KO mice might thus serve as a model in which diet/environmental factors and microbes may be controlled and investigated in relation to intestinal inflammation. Potential molecular mechanisms include defective transport of inflammatory mediators and/or phospholipid translocation from one side to the other of the cell membrane lipid bilayer by ABC transporters affecting inflammatory response and/or function of tight junctions, phagocytosis and vesicle trafficking. Also, diet and microbes give rise to molecules which are potential substrates for the ABC transporters and which may additionally affect ABC transporter function through nuclear receptors and transcriptional regulation. Another critical role of ABCB1 was suggested by the finding that ABCB1 expression identifies a subpopulation of pro-inflammatory Th17 cells which were resistant to treatment with glucocorticoids. The evidence for the involvement of ABCC2 and ABCG2 in colonic pathophysiology was weak.

CONCLUSION: ABCB1, diet, and gut microbes mutually interact in colonic inflammation, a well-known risk factor for CRC. Further insight may be translated into preventive and treatment strategies.

Key words: ATP-binding cassette transporters; Colorectal cancer; Intestinal; Inflammatory bowel disease; Inflammation; Adenoma-carcinoma sequence

Core tip: Recently, human studies reported that changes in the levels of ATP-binding cassette (ABC) transporters were early events in the adenoma-carcinoma sequence leading to colorectal cancer. A link between ABCB1, high fat diet and gut microbes in relation to colitis was suggested by the animal studies. The *Abcb1* KO mice might thus serve as a model in which diet/environmental factors and microbes may be controlled and investigated in relation to intestinal inflammation. Such strategy may provide insight which can be translated into preventive and treatment strategies to benefit the patients.

Andersen V, Svenningsen K, Knudsen LA, Hansen AK, Holmskov U, Stensballe A, Vogel U. Novel understanding of ABC transporters ABCB1/MDR/P-glycoprotein, ABCC2/MRP2, and ABCG2/BCRP in colorectal pathophysiology. *World J Gastroenterol* 2015; 21(41): 11862-11876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i41/11862.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i41.11862

INTRODUCTION

Colorectal cancer (CRC) constitutes the third most common cancer in the world and the second leading cause of cancer-related deaths. The number of cases is increasing and has been estimated to raise from 1.4 million cases in 2012 to 2.4 million cases in 2035 worldwide[1]. Early detection of CRC is important as early treatment has been associated with improved outcomes and saved lives[2]. Therefore, population screening programs have been initiated in a number of countries such as the United Kingdom, Australia, Holland and Denmark[3-6]. The fecal occult blood test (FOBT) is the most widely used for population screening[7] and individuals with a positive FOBT are referred for an endoscopic investigation of the colonic mucosa thereby enabling the sampling of biopsies from the colonic mucosa.

Recently, a major part of research had focused on improving prognosis and treatment selection in CRC[8-10]. Another approach could be to prevent the development of cancer in subgroups of patients with high risk, *i.e.,* secondary prevention. Thus, the molecular evaluation of the (unaffected) colonic mucosa from the patients undergoing an endoscopic evaluation could potentially stratify the patients according to their risk of developing CRC. Our recent findings indicate that even healthy looking mucosa as determined by histology may contain a significantly elevated level of immune response proteins[11]. Biomarkers potentially predicting the disease risk among selected patient groups could improve the efficiency of the screening programs and patient care. Furthermore, they have the potential to dramatically alter the established patient care pathways as follow-up of the patients may be tailored according to their individual risk and thereby the organization and use of resources of the health care system.

CRC develops in the colonic mucosa which is highly affected by the metabolic activities in the intestinal lumen. The dietary items reaching the colon are digested by the commensal bacteria giving rise to various substrates which may prevent, initiate or promote colorectal cancer development[12]. Thus, in order to understand the processes leading to CRC we need to take into account the delicate interactions between dietary intake, activity of the commensal bacteria and host factors.

We recently reported that low *ABCB1* and *ABCG2* gene transcription levels and high *ABCC2* levels are early events in the colorectal adenoma-carcinoma sequence[13,14] suggesting that changes in expression levels of the ATP binding cassette (ABC) transporter proteins [EC 3.6.3.44] precede cancer development. In addition, inflammatory bowel disease (IBD) may be

a risk factor for the development of CRC[8]. Therefore, we wanted to discuss the current understanding of how these ABC transporters may affect intestinal inflammation and carcinogenesis, how they may potentially interact with the environment such as diet and gut microbes, and whether this knowledge may be utilized for improved treatment care strategies.

MATERIALS AND METHODS

Literature search was conducted on PubMed using combinations of the following terms: ABC transporters, ATP binding cassette transporter proteins, inflammatory bowel disease, ulcerative, colitis, Crohn's disease, colorectal cancer, colitis, intestinal inflammation, intestinal carcinogenesis, ABCB1/P-glycoprotein (P-gp/CD243/MDR1), ABCC2/multidrug resistance protein 2 (MRP2) and ABCG2/breast cancer resistance protein (BCRP), *Abcb1/Mdr1a*, *abcc2/Mrp2*, *abcg2/Bcrp*, knock-out mice, tight junction, membrane lipid function.

RESULTS

ABC family of transporters; ABCB1, ABCC2, and ABCG2

The large family of ABC transporter proteins is highly conserved through evolution and extensive sequence and protein homology is shared between numerous bacterial and eukaryotic ABC transport proteins[15]. The ABC proteins are found in the cell membranes and intracellular organelles and the ABC family members exert multiple different functions depending on the cellular context[16].

The ABCB1, ABCC2, and ABCG2 transporters, encoded by *ABCB1*, *ABCC2*, and *ABCG2,* respectively, are located in the apical cell membrane of epithelial and endothelial interfaces within the intestine, testis, kidneys, liver, brain, and placenta[17-20]. Thereby, they exert barrier functions influencing absorption, distribution, excretion, and toxicology (ADME-Tox) of exogenous substrates with potential impact on inflammation and carcinogenesis[21-25]. ABCB1 and ABCG2 transporters have also been identified on haematological cells[20,26,27]. Whereas ABCB1 has been extensively studied in relation to the gastrointestinal system[28], less is known for ABCC2 and ABCG2[29].

No monogenic diseases have been identified involving *ABCB1* and *ABCG2*[30,31], but several different mutations in *ABCC2* have been observed in patients with Dubin-Johnson syndrome, an autosomal recessive disorder characterized by conjugated hyperbilirubinemia[32].

Nuclear receptors such as aryl hydrocarbon receptor (AHR), pregnane x receptor (PXR, NR1I2), vitamin D receptor (VDR, NR1I1), and constitutive androstane/activated receptor (NR1I3) are activated by a wide variety of exogenous and endogenous factors including diet, heavy metals, gut microbes, carcinogens and inflammation[33,34] (reviewed in[35]). These nuclear receptors may be involved in the transcriptional regulation of ABC transporters[34,36-40] as are the transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP-1)[41], and Wnt signaling transcription factor TCF4[42]. Furthermore, ABCB1 undergoes several posttranslational modifications (PTMs)[43,44] which have been shown to affect the stability of ABCB1 and/or substrate transport specificities[45]. ABCB1 is a 170-180 kDa glycoprotein with N-linked glycosylation at residues Asp^{91}, Asp^{94} and Asp^{99}. ABCB1 and ABCC2 have two ATP-binding sites and two six-transmembrane domains in a symmetric structure whereas ABCG2 is a half-transporter and have one ATP binding site and one six-transmembrane domain.

ABC transporter substrates include many diverse endogenous and exogenous molecules including amino acids, peptides, metabolites, vitamins, fatty acids, steroids, phospholipids, conjugated organic anions, and dietary and environmental carcinogens, pesticides, metals, metalloids, lipid peroxidation products and drugs[22-24]. Substrate overlap has been reported between the ABCB1, ABCC2, ABCG2, and especially between ABCC2 and the basolaterally located ABCC1[23,29]. Specific substrates and their potential role in ABC transporter related gut inflammation will be discussed later in this review.

Inflammation is a key factor underlying the development of CRC

CRC is a heterogeneous disease complex with environmental, genetic and host factors involved in the aetiology[46,47]. Inflammation is a risk factor for CRC[48-50] and accordingly, a subset of patients with IBD[51,52] [with the two main forms ulcerative colitis (UC) and Crohn's disease (CD)] characterised by long-term and extensive colitis are at high risk of CRC[53,54]. The incidences of both CRC and IBD are rising[1,55], which point to important roles of environment factors.

The intestinal mucosa is by far the body's largest surface exposed to and interacting with environmental factors. The intestinal epithelium and the mucus form a barrier against luminal antigens and invading microbes[56,57]. Microbial sensing by intestinal epithelium cells and local innate lymphoid cells (ILCs) through pattern recognition receptors (PRR) leads to secretion of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin 6 (IL-6), and IL-17[58,59], cytokines which have been related to IBD and CRC[60]. Activation of PRR stimulates autophagocytic networks[61,62]. Also, activation of the innate immune system may result in activation of the adaptive immune response with T cell involvement; Th1, Th2 and Th17 cells characterised by secretion of their signature cytokines INF-γ, IL-4, IL-17, respectively, whereas Tregs (and to a lesser degree Th2), in contrast, are characterised by their production of the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β)[63,64]. The

Table 1 The *ABCB1*, *ABCC2* and *ABCG2* mRNA and protein levels in intestinal tissue from patients with ulcerative colitis

		Controls	Inactive disease				Active disease				Ref.
		Colon	Colon	*P* value	Rectum	*P* value	Colon	*P* value	Rectum	*P* value	
Gene	*ABCB1*[1]	1 (ref)	NA	NS	NA		22%	< 0.001	34%	< 0.01	[72]
	ABCC2[1]	1 (ref)	NA	NS	NA		NA	NS	NA	NS	[72]
	ABCG2[1]	1 (ref)	NA	NS	NA		11%	< 0.001	16%	< 0.001	[72]
Array	*ABCB1*[2]	287	-1.5								[40]
	ABCC2[2]	81	-8.6								[40]
Protein	ABCG2[3]	100 (9/9)	80 (53/67)				24 (13/54)	0.01			[73]

[1]The *ABCB1*, *ABCC2* and *ABCG2* mRNA levels in colon and rectum tissue from patients with ulcerative colitis in remission (n = 17) or with active disease (n = 16) compared to the levels in colon tissue from healthy controls (n = 17). mRNA levels are normalised to the villin mRNA level. *P* values compared to the expression in the controls; [2]Microarray analyses of pooled cRNA from uninflamed colonic tissue from 4 patients with UC and 4 control subjects. Fold change expression in colon tissue compared to controls. Statistically significant expression levels of *ABCB1* were found in UC patients compared to controls by RT-PCR analyses using 18S RNA as internal control ($P < 0.05$); [3]Quantitative immunohistochemistry of formalin-fixed paraffin-embedded (FFPE) colonic biopsies from 9 healthy individuals and 36 patients with ulcerative colitis. The values are n % (samples with positive staining/total number). *P* value for active colitis compared to controls and inactive colitis, respectively. NA: Not available; NS: Not significant.

role of the Th17-associated cytokines in animal models of colitis[65], IBD[66] and CRC[67] have been in focus the recent years and it has been suggested that Th17 cells may have evolved to combat bacterial and fungal infections *via* orchestration of the neutrophil inflammatory response[63]. However, this seems to be a simplistic view[68] and more T cell subsets with as yet unclarified functions in IBD and CRC have been identified these years[69-71].

ABC transporters, IBD and CRC

Englund *et al*[72] found significantly lower levels of both *ABCB1* and *ABCG2* mRNA in colon and rectal biopsies from 16 patients with active UC compared to healthy individuals whereas the levels did not differ between UC patients in remission and healthy controls (Table 1). The authors also reported lower ABCB1 and ABCG2 levels in colon from patients with active inflammation compared with controls[72]. Langmann *et al*[40] reported low levels of *ABCB1* and *ABCC2* mRNA in biopsies from colon adjacent to inflammation from patients with UC compared to the levels in controls. In contrast, Deuring *et al*[73] reported similar levels of *ABCG2* mRNA in intestinal biopsies from healthy individuals, patients in remission and patients with active inflammation but dramatically reduced levels of ABCG2 in IBD patients with active inflammation when compared to patients in remission or healthy controls using quantitative immunohistochemistry (Table 1). These observations suggest that the low levels of ABCG2 observed in inflamed colon were caused by posttranscriptional processes[73]. The study also found inflamed colon to contain high levels of the endoplasmic reticulum (ER)-stress marker GRP78 and *in vitro* they found nitric oxide induced ER-stress to impair ABCG2 function[73]. The authors therefore suggested that incorrect protein folding caused by inflammation-induced ER dysfunction may lead to low levels of ABCG2 in inflamed colon of IBD patients[73,74].

The role of ABC transporters has also been investigated in relation to CRC (Table 2). As previously mentioned, low levels of *ABCB1* in colon was found to be an early event that preceded malignancy[13]. Similarly, in another study using the same cohort low levels of *ABCG2* and high levels of *ABCC2* mRNA were found in both colon adenomas and carcinomas compared to morphological normal tissue surrounding the cancer tissue, and compared to levels in tissue from healthy individuals[14]. Taken together, the studies suggest that changed expression levels of the ABC transport proteins may be early events in the development of IBD and CRC.

Genetically determined variation in ABC transporters has been investigated in relation to risk of developing IBD[75-79] and CRC[80-82] with varying results[83-85]. In particular the polymorphisms *ABCB1* C1236T, G2677T/A, and C3435T have been investigated. These polymorphisms are in linkage disequilibrium. Haplotype frequencies vary among ethnic groups and the CGC and TTT haplotypes are frequent among Caucasians[86]. The synonymous C3435T polymorphism was reported to cause changes in protein folding due to ribosome stalling caused by impaired interaction between the tRNA and the chaperone protein that aids the folding process at the ribosome[86] which resulted in altered transporter function[87]. A recent meta-analysis found that the *ABCB1* C3435T polymorphism (rs1045642) was associated with risk of UC, but not with CD[84]. In relation to CRC, a large case-control analysis of a Czech and two German cohorts of 4677 cases in total found no indications of a strong role of *ABCB1* in CRC[88] which was in accordance with a meta-analysis (not including the above study)[85]. A prospective study based on a Danish cohort found that two *ABCB1* polymorphisms, including the C3435T polymorphism, were associated with CRC risk[82]. Furthermore, these two polymorphisms were found to interact with meat intake in relation to risk of CRC. Only few studies of *ABCC2* and *ABCG2* polymorphisms as risk factors for IBD and CRC have been performed. No strong indications that genetic variation in *ABCC2* or *ABCG2 per see* is associated with IBD or CRC were

Table 2 The *ABCB1*, *ABCC2* and *ABCG2* mRNA levels in intestinal tissue from patients with adenomas and colorectal cancer and healthy individuals

	Unaffected tissue	*P* value[1]	Adenomas/carcinomas	*P* value[1]	*P* value[2]	Ref.
ABCB1						[13]
Healthy individuals	0.012 ± 0.008					
Mild/moderate dysplasia cases	0.009 ± 0.004	NS	0.005 ± 0.004	< 0.050	< 0.001	
Severe dysplasia cases	0.009 ± 0.030	NS	0.003 ± 0.002	< 0.050	< 0.001	
Cancer patients	0.009 ± 0.014 (distant)	< 0.05	0.003 ± 0.005	< 0.001	< 0.001	
	0.007 ± 0.009 (adjacent)	< 0.05			< 0.010	
ABCC2						[14]
Healthy individuals	5.35 ± 3.24					
Mild moderate dysplasia cases	4.62 ± 4.79	0.081	6.68 ± 6.77	0.87	0.037	
Severe dysplasia cases	6.66 ± 8.47	0.880	10.18 ± 11.52	0.27	0.240	
Cancer patients	28.06 ± 68.84 (distant)	0.036	87.50 ± 270.21	0.0046	0.0037	
	11.44 ± 25.58 (adjacent)	0.690			< 0.0001	
ABCG2						[14]
Healthy individuals	718.06 ± 761.24					
Mild moderate dysplasia	732.85 ± 2305.28	0.550	56.02 ± 118.42	< 0.0001	< 0.0001	
Severe dysplasia	448.02 ± 195.34	0.840	76.31 ± 102.63	< 0.0001	< 0.0001	
Cancer patients	6679 ± 58353 (distant)	0.080	98.41 ± 476.36	< 0.0001	< 0.0001	
	1302 ± 10090 (adjacent)	0.011			< 0.0001	

[1]*P* values for comparison of the expression levels in tissue from healthy individuals adjusted for age and gender. Samples were available for 18 healthy controls, 88-94 patients with mild/moderate dysplasia, 12 with severe dysplacia, and 121-122 patients with CRC; [2]*P* value for the comparison of the expression levels in morphologically unaffected and affected tissue from the same individual using Paired Student's *t*-test. All values are mean ± SD. *ABCB1* mRNA levels are normalised to the *β-actin* mRNA level. *ABCC2* and *ABCG2* mRNA levels are normalised to 18S RNA levels. Matching samples were available from ABCG2: 66-75 cases with mild-moderate dysplasia, 11 cases with severe dysplasia, and 63-80 and 66-99 CRC cases (distant unaffected tissue, and adjacent unaffected tissue, respectively). NS: Not significant.

found[80,81].

ABC transporters and colitis and dysplasia in animal models

The *Abcb1/Mdr1a* knock-out (*Mdr1a* KO) mouse, in which the gene corresponding to the human intestinal *ABCB1* gene has been deleted[89,90], has been utilized as an animal model of colitis[91-95]. The colitis is characterized by histological changes and high levels of the cytokines INF-γ, TNF-α, IL-1β, IL-6 and IL-17 thus resembling the findings in UC patients. The classical study by Panwala *et al*[91] reported that a proportion of *Mdr1a* KO mice developed colitis when exposed to commensal gut bacteria. The development of spontaneous colitis was prevented if the mice were maintained germfree. Also, spontaneous colitis and active inflammation was resolved by oral treatment with a mixture of streptomycin, neomycin, bacitracin, and amphotericin. These findings highlight an important role of bacteria in the initiation and perpetuation of colitis in the *Mdr1a* KO mouse[91]. Since then, the finding that lack of *Mdr1a* confers risk of colitis has been replicated by others[94-98]. Furthermore, a proportion of the *Mdr1a* KO mice dual-infected with Helicobacter species (*H.bilis* and *H. hepaticus*) developed dysplasia[99].

One study found redused in the diversity and total number of bacteria in *mdr1a* KO mice compared to wildtype mice. These alterations were found to precede and associate with the development of inflammation[95]. Another study reported changes in colonic gene expression which also preceded disease development[98]. High expression of INF-γ was found in histologically normal colonic tissue from *Mdr1a* KO mice and the change preceded a high expression of the inflammatory cytokines IL-1β, IL-6, TNF-α, increased colonic permeability, and histologically determined colon inflammation[98]. Yet, another study found a high level of the pro-inflammatory cytokine IL-17 in colon from the *Mdr1a* KO mice model[92]. INF-γ expression has been associated with reduced intestinal barrier function due to effects on tight junction proteins[96]. Also, one study suggested that impaired intestinal barrier function contributed to the development of colitis in *Mdr1a* KO mice. In this study, high permeability of FITC-dextran (4.4 kDa) and horseradish peroxidase (44 kDa) was found in colon tissue mounted in Ussing chambers and *in vivo*, high bacterial translocation to lymphoid tissue including increased trabecular infiltrate with neutrophils were found[94]. These changes were observed prior to onset of colitis. Furthermore, decreased phosphorylation of tight junction proteins including occludin was observed[94]. Thus, inflammation and the following high INF-γ expression may contribute to the loss of barrier function which has been observed in the *Abcb1* KO mice.

High fat diet-induced obesity increases the frequency and severity of colitis in the *mdr1a* KO mice[100]. Wildtype mice feeding either high-fat diet or low fat diet did not develop colitis[100]. In contrast, specific pathogen free *Mdr1a* KO mice fed high fat diet had a higher frequency and more severe colitis compared to those who were fed a low fat diet[100]. Although

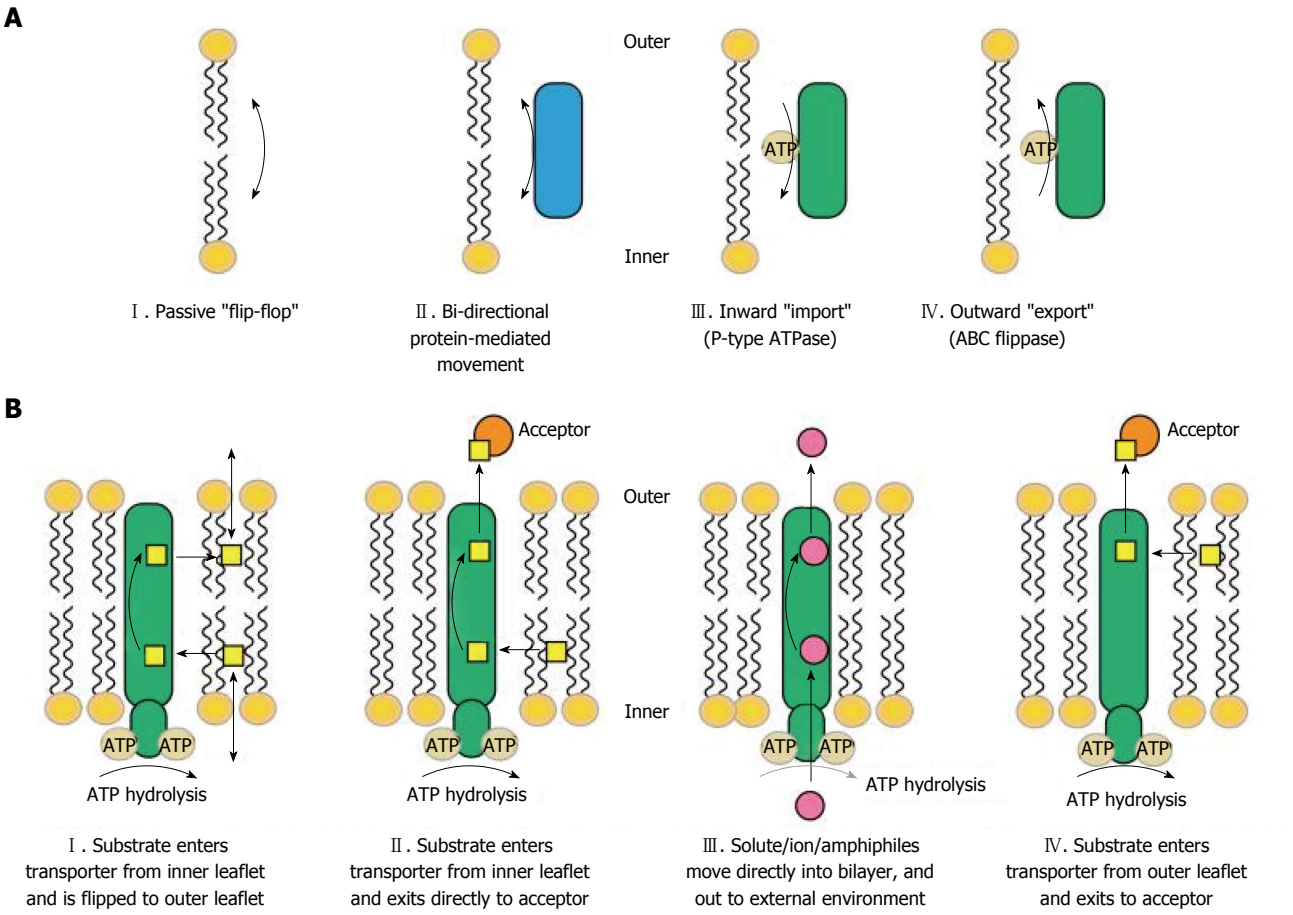

Figure 1 The models presented are from Tarling *et al*[16]. A: Lipids can move across the membrane bilayer by multiple mechanisms. Four mechanisms are proposed here: (1) membrane lipids passively diffuse or "flip-flop" from one leaflet of the bilayer to another; (2) bi-directional movement of lipids from one membrane leaflet to another is enhanced by proteins present in the membrane bilayer; (3) P-type ATPases mediate the movement of specific lipids (phospholipids) from the outer leaflet of the membrane bilayer; and (4) ABC transporters/flippases mediate the "outward" movement of specific lipids (phospholipids/cholesterol) from the inner leaflet to the outer leaflet of the membrane bilayer; B: Mechanisms of substrate recognition and transport by ABC transport proteins: (1) substrates enter the transporter from the inner leaflet and are flipped to the outer leaflet where they can exit the membrane bilayer; (2) as in (1) but the substrate exits the transporter directly to an exogenous acceptor; (3) solute/ions/amphiphiles move directly into the bilayer, through the transporter protein and out to the external environment; and (4) substrates enter the transporter from the outer leaflet and exits to an acceptor molecule.

the microbiota was not investigated in this study, the authors concluded that the diet and potential diet-induced changes in microbiota was not sufficient to induce colitis in the mice but that additional host genetic factors are required before the high fat diet is a risk factor for colitis[100].

Impaired immune system may also be involved in the aetiology of colitis in the *Mdr1a* KO mice model. In mice, regulatory T cells (Tregs) characterised by the expression of the transcription factor Foxp3[101] are considered to down-regulate effector T cells that react to microbial or other gastrointestinal antigens. In the study by Tanner *et al*[97], they also found that there appeared to be fewer Tregs present in intestine from *mdr1a* KO mice and that these Tregs were unable to effectively suppress TNF-α induced colitis. These results are in accordance with the notion that inflammation primarily is initiated by the innate immune system.

In contradiction to the findings in the *Mdr1a* KO mice model, *Abcc2/Mrp2* KO and *Abcg2/Bcrp1* KO mice were found to be phenotypically normal under standard housing conditions[102,103].

The molecular mechanisms of ABC transporters may involve phospholipid transport

Cellular processes such as phagocytosis, apoptosis, cytokine release, vesicle formation and tight junction function require cell membrane budding and curvature and therefore, different composition of the inner and outer side of the lipid bilayer forming the cell membrane (Figure 1)[104]. Translocation of phospholipids between the two sides of the lipid bilayer within the cell membrane is therefore important for generating such differences. ABCB1, ABCC2, and ABCG2 have been found to translocate various phospholipid membrane components; cholesterol, sphingomyelin, and other glycosphingolipids suggesting that ABC transporters are important for regulating the budding of the membrane function[15,16,105,106]. Furthermore, the cellular processes also require cell cytoskeleton anchoring through specialised domains[107]. ABCB1 has been

found to be associated with such domains[106,108,109]. Other phospholipid transporters such as scramblases, P_4-ATPases and additional members of the ABC transporter family, are reviewed in[15].

In vitro studies of rat kidney and Sertoli cells support the involvement of ABC transporters in tight junction function and apoptosis[110,111]. At the Sertoli cell blood-testis barrier, ABCB1 was found to co-localise with occluding, claudin-11 and junction adhesion molecule A[110]. Knockdown of *Abcb1* (*Abcb1a* and *Abcb1b*) by RNAi in rat Sertoli cell cultures led to a decline of claudin-11, internalisation and degradation of occluding, and disruption of tight junction barrier function[110]. Another study found that ABCB1 decreased apoptosis by decreasing the availability of a precursor of ceramide[111], an intracellular signalling molecule involved in apoptosis induced by TNF-α and other apoptotic stimuli[106,108]. However, the functions of the ABC transporters may be tissue specific and therefore the results may not apply for intestinal conditions.

The molecular mechanisms of ABC transporters may be related to the transport of other substrates

Figure 1 shows mechanisms of substrate recognition and transport by ABC transporters[16]. An *in vitro* study by Pawlik *et al*[112] on cultured peripheral blood mononuclear cells PBMC from healthy individuals found that stimulation with phytohaemagglutinin (PHA) leads to secretion of IL-2, IL-4, IL-6, IL-10, INF-γ, and TNF-α[112]. Furthermore, secretion of IL-2, IL-4, INF-γ, and TNF-α was inhibited by anti-MDR1 specific antibody whereas secretion of IL-6 and IL-10 was unaffected. In a similar study, blockade of ABCC1 by anti-MRP1 specific antibodies led to reversible abrogated cytokine secretion of IL-10, TNF-α, IL-4 and INF-γ[113]. However, another study using splenocytes from *Mdr1a* KO mice found that IL-2, IL-4, IL-10, and INF-γ secretion was independent of ABCB1. The authors suggested that ABCB1 may not be required for secretion of these cytokines because they contain a signal sequence designating the cytokines for secretion from the cells[114]. Yet, a further *in vitro* study by Pawlik *et al*[115] on cultured PBMC, this time from 72 healthy *ABCB1* genotyped individuals was conducted. The cultured cells were stimulated with PHA and cytokines were measured in the supernatant. The authors found significantly lower concentration of IL-2, IL-4, INF-γ, and TNF-α, and unchanged concentration of IL-6 and IL-10 in cultured cells from individuals with *ABCB1* C3435T TT genotypes compared to CC genotypes[115]. Also, ABCB1 blockade by the antagonist PSC833 resulted in impaired IL-12 secretion by antigen presenting cells from peripheral blood from healthy human volunteers suggesting that functional ABCB1 is required for IL-12 secretion in these cells[116]. As previously mentioned, cytokines and chemokines are important modulators of intestinal inflammation and carcinogenesis[108,117]. Additionally, ABCB1, ABCC2, and ABCG2 also transport bioactive lipids[15,16,105]. The levels of the ABCB1 substrate platelet-activating factor[117-119] have been found to be high in intestinal mucosa from CD patients[120]. PAF has been reported to regulate the function of tight junctions[121] and to activate human neutrophils to extrusion of neutrophil extracellular traps (NETs) mediating extracellular capture and killing of bacteria[122,123]. Also, ABCB1 has been reported to transport steroids, mineralocorticoids, androgens and oestrogens[106]. Interestingly, the ABC substrate testosterone was found to be a key mediator of autoimmune responses in the non-obese diabetic mouse model of type 1 diabetes[124]. Whether a similar phenomenon contributes to the observed male preponderance in *Mdr1a* KO IBD mouse model has not been studied as far as we know[94]. ABCG2 transport the anti-inflammatory butyrate, a product of bacterial digestion of dietary fibres, and phytoestrogen from vegetables[125,126]. ABCC2 has been reported to transport the pro-inflammatory signalling molecules leukotriene (LT) B_4 and LTC_4 involved in dendritic cell migration and CRC, and, furthermore, various diet- and smoke-derived carcinogens[127-131]. Sulfasalazine and 5-aminosalicylic acid (5-ASA, mesalazine) are used for treatment and prevention of UC flares[132]. ABCG2 is regarded as being the main transporter of sulfasalazine[133,134] and ABCG2 activity has been suggested as having impact on sulfasalazine treatment efficacy in patients with rheumatoid arthritis (RA)[135,136].

ABCB1 expression on T cells may identify pro-inflammatory Th17 cells

One study utilised ABCB1 expression to identify human Th17 cells with a unique pro-inflammatory transcriptional signature[20]. This novel subset of Th17 cells, MDR1-positive Th17 cells, was identified by fluorescence activated cell sorting (FACS) analysis of PBMC from healthy individuals. Compared to MDR1-negative Th17 cells, the MDR1-positive Th17 cells were characterized by a high production of pro-inflammatory Th1 (INF-γ) and Th17 (IL-17A, IL-17F, and IL-22) cytokines and low levels of anti-inflammatory cytokines such as IL-10 upon stimulation[20]. In contrast to the MDR1-negative T cells, the MDR1-positive T cells were resistant to treatment with glucocorticoids. Thus, MDR1-positive T cells from healthy humans were enriched two- to three-fold during culturing of peripheral blood memory T cells in the presence of glucocorticoids[20]. Furthermore, in a small study of 3-5 CD patients, MDR1-positive Th17 cells (assessed as percent of the total number of memory cells) were enriched both in non-inflamed and inflamed gut tissue compared to blood levels[20]. High mRNA levels of IFN-γ, IL23R, and TNF were found in MDR1-positive Th17 cells compared to MDR1-negative Th17 cells following FACS-sorting of mononuclear cells from gut tissue from two CD patients[20].

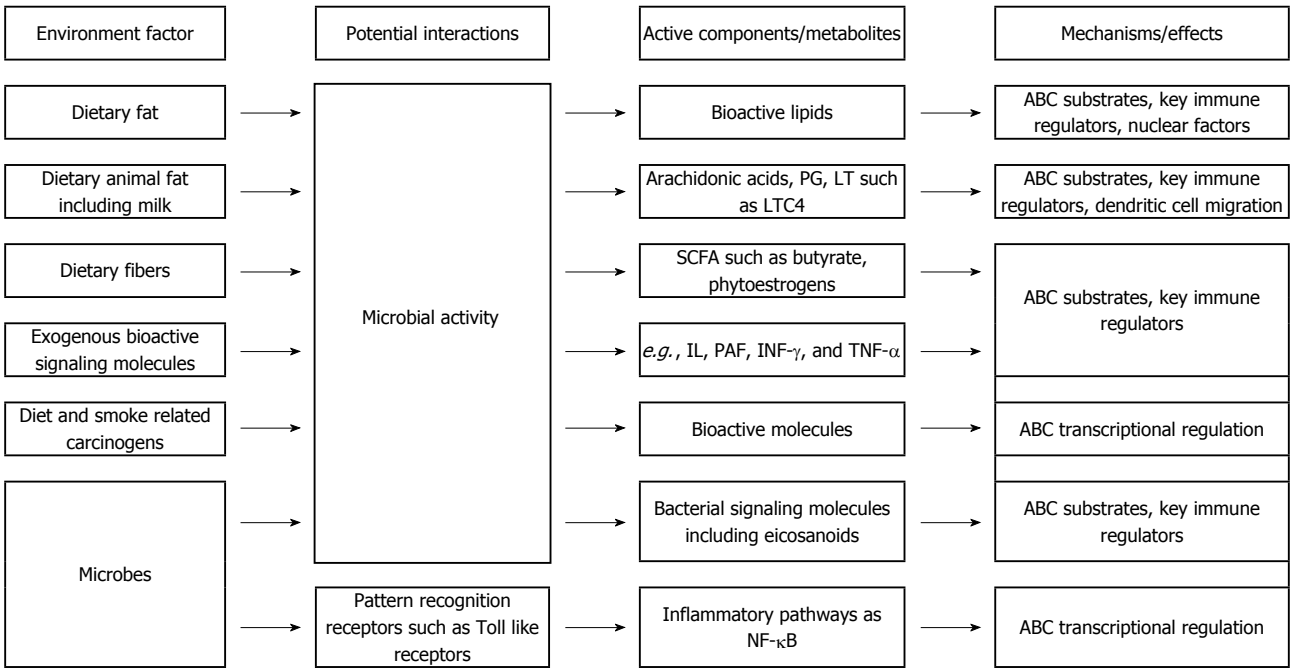

Figure 2 Proposed mechanisms for the involvement of ABC transporters in intestinal inflammation.

DISCUSSION

The ABC transport proteins may confer a link between the environment and intestinal inflammation and potentially intestinal carcinogenesis *via* intestinal inflammation[48-50,137,138] . Diet affects risk of CRC[1], the course[139-143] and risk of IBD [144-148] (reviewed in[149-153]). Diet affects gut microbial composition[154,155] and both diet and intestinal microbes affect intestinal inflammation[156,157] and carcinogenesis[12,158-161].

A link between ABCB1, diet and the gut microbes in relation to colitis is suggested by the animal studies. High fat diet increases the frequency and severity of colitis in specific pathogen-free *Abcb1* KO mice[100]. Undigested dietary items reaching the colon are digested by commensal bacteria thereby providing the host with valuable energy, essential vitamins, fatty acids etc. Dietary fibre from grains, fruit and vegetables is converted into short-chain fatty acids (SCFA) which represent important key regulators of the immune system[12]. The gut microbiome in active IBD is characterised by decreased microbial diversity with a decreased number of Firmicutes[162]. Low abundance of the *Clostridium* and *Bacteroides* species which preferentially produce butyrate and other SCFA may result in low production of SCFA[163]. High intake of meat which is a rich source of sulphur may lead to the formation of hydrogen sulphide by bacterial fermentation[12] which, at least theoretically, may be aggravated by high intake of milk fat which was found to favour the presence of the sulphate-reducing bacteria *Bilophila wadsworthia* in mice[157]. Also, intake of animal fat may give rise to arachidonic acid which is converted into *e.g.,* prostaglandins and leukotrienes[12]. Some of these molecules are ABC transporter substrates including dietary pro- and anti-inflammatory molecules, bioactive lipids, and bacterial derived molecules[125,126]. Figure 2 shows potential mechanisms of the involvement of ABC transporters in inflammation. In addition, diet and other environmental factors may impact the transcriptional regulation of ABC transporters through effects on nuclear receptors and transcription factors leading to changes of the ABC transporter activity thereby affecting IBD and CRC. The ABC transporters may impact IBD and CRC through their transport of various substrates thereby affecting underlying biological mechanisms involved in intestinal inflammation (Figures 1 and 2).

ABC transporter polymorphisms have been evaluated in relation to development of IBD and CRC with inconsistent results. These studies are based on the hypothesis that genetic variations are associated with functional changes in ABC activity and/or specificity. It has been suggested that genetic diversity of the *ABCB1* gene among various ethnicities may contribute to the varying results in candidate gene studies[164,165]. In addition, *ABCB1* polymorphisms may only be associated with risk of CRC in populations with a relevant dietary exposure[166]. This aspect may be exemplified by the finding of an interaction between meat intake and the gene *NFKB1* encoding NFκB p50 in a Danish cohort[137]. This interaction may explain the finding that the *NFKB1* polymorphism was associated with risk of CRC in a Swedish cohort but not in a Chinese cohort[167]. Meat intake are higher in Denmark and Sweden compared to China[168]. Therefore, *NFKB1* was identified as a risk gene in the Danish and Swedish high meat intake cohorts but not in the Chinese low

meat intake cohort. A detailed assessment of the diet seems to be important for assessing the roles of ABC polymorphisms. Thus, future studies should focus on studying large cohorts with well-defined and relevant prospectively sampled environmental exposures in order to identify underlying IBD and CRC disease mechanisms.

Due to the many confounding parameters, potential causality cannot be evaluated through molecular epidemiological studies. Studies using animal models, where a range of parameters can be controlled are therefore needed for establishing causality. Germfree mice do not develop colitis. Although germfree mice are not exposed for living bacteria they will meet dietary derived microbial antigens which could activate PRR in the mucosa and induce inflammation. Inflammation, however, has not been observed in the germfree mice. Moreover, colitis can be prevented by antibiotics in conventionally housed, specific pathogen-free, mice. These findings suggest that microbial derived antigens are not sufficient to trigger colitis but that living microbes are needed and may thus point to potential mechanisms such as microbial derived metabolites, signalling peptides and extracellular vesicles[169,170]. Indeed, gut microbial derived metabolites were found to affect the balance between pro- and anti-inflammatory cells in mice[171]. These metabolites may be absorbed into the blood and thereby affect distant organs. Gut microbes have been reported to affect the immune system, in particular the Th17 pathway, in various autoimmune mouse models[172-176]. Some studies, but not all[177], indicate a similar mechanism in humans which might also associate with human autoimmunity[178-180]. Also, bacterially derived fatty acids and other relevant metabolites should be investigated in the *Abcb1* KO mice like it has been done in male C57BL/6 (B6) mice[171]. The *Abcb1* KO mice might provide a model, in which the interplay of environment factors, diet, and microbes can be controlled and investigated. Due to important differences of human and murine immune systems, the translational value of results obtained from the mouse model need also to be evaluated through human data.

The finding that presence of ABCB1 on immune cells could be used to identify pro-inflammatory Th17 cells may have important clinical implications as glucocorticoids are a mainstay in the treatment of serious flares of IBD[181] and since a large proportion (20%-30%) of patients are resistant to glucocorticoid treatment[182]. Thus, high ABCB1 mediated drug efflux may lead to decreased intracellular drug concentrations in target cells [183, 184] and thereby confer glucocorticoid treatment resistance. Likewise, ABCG2 activity may affect efficacy of treatment with sulfasalazine. Further evaluation of the roles of ABC transporters in treatment response in IBD is warranted.

In conclusion, results from animal and human studies indicate that ABCB1, diet, and gut microbes mutually interact in colonic inflammation. Diet and microbes may give rise to molecules which are substrates for the ABC transporters and may additionally affect ABC transporter function through *e.g.,* nuclear receptors and transcriptional regulation. The *Abcb1* KO mice might provide a model in which these factors can be controlled and investigated. Such strategy may provide insight which can be translated into preventive and treatment strategies to benefit the patients. The evidence for the involvement of ABCC2 and ABCG2 in colitis was weak.

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COMMENTS

Background

Colorectal cancer (CRC) constitutes the third most common cancer in the world and the second leading cause of cancer-related deaths. The number of cases is increasing and has been estimated to raise from 1.4 million cases in 2012 to 2.4 million cases in 2035 worldwide. Early detection of CRC is important as early treatment has been associated with improved outcomes and saved lives. Therefore, population screening programs have been initiated in a number of countries such as the United Kingdom, Australia, Holland and Denmark. The fecal occult blood test (FOBT) is the most widely used for population screening and individuals with a positive FOBT are referred for an endoscopic investigation of the colonic mucosa thereby enabling the sampling of biopsies from the colonic mucosa.

Research frontiers

Recently, a major part of research had focused on improving prognosis and treatment selection in CRC. Another approach could be to prevent the development of cancer in subgroups of patients with high risk, *i.e.,* secondary prevention. Thus, the molecular evaluation of the (unaffected) colonic mucosa from the patients undergoing an endoscopic evaluation could potentially stratify the patients according to their risk of developing CRC. Recently, human studies by authors reported that changes in the levels of ABC transporters were early events in the adenoma-carcinoma sequence leading to CRC. These findings indicate that even healthy looking mucosa as determined by histology may contain a significantly elevated level of immune response proteins.

Innovations and breakthroughs

The authors recently reported that low ABCB1 and ABCG2 gene transcription levels and high ABCC2 levels are early events in the colorectal adenoma-carcinoma sequence suggesting that changes in expression levels of the ATP binding cassette (ABC) transporter proteins [EC 3.6.3.44] precede cancer development. In addition, inflammatory bowel disease (IBD) may be a risk factor for the development of CRC. Therefore, the authors wanted to discuss the current understanding of how these ABC transporters may affect intestinal inflammation and carcinogenesis, how they may potentially interact with the environment such as diet and gut microbes, and whether this knowledge may be utilized for improved treatment care strategies. A link between ABCB1, high fat diet and gut microbes in relation to colitis was suggested by the animal studies. The Abcb1 KO mice might thus serve as a model in which diet/environmental factors and microbes may be controlled and investigated in relation to intestinal inflammation. Such strategy may provide insight which can be translated into preventive and treatment strategies to benefit the patients.

Applications

Biomarkers potentially predicting the disease risk among selected patient groups could improve the efficiency of the screening programs and patient care. Furthermore, they have the potential to dramatically alter the established

patient care pathways as follow-up of the patients may be tailored according to their individual risk and thereby the organization and use of resources of the health care system.

Peer-review

Congratulations to the authors for their review on ABC transporters ABCB1/MDR/P-glycoprotein, ABCC2/MRP2, and ABCG2/BCRP in colorectal pathophysiology. It is certain that this paper will be very inspiring in this field. Personally recommend it to be accepted.

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SYSTEMATIC REVIEWS

Nomograms for colorectal cancer: A systematic review

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Abstract

AIM: To assist in the selection of suitable nomograms for obtaining desired predictions in daily clinical practice.

METHODS: We conducted electronic searches for journal articles on colorectal cancer (CRC)-associated nomograms using the search terms colon/rectal/colorectal/nomogram. Of 174 articles initially found, we retrieved 28 studies in which a nomogram for CRC was developed.

RESULTS: We discuss the currently available CRC-associated nomograms, including those that predict the oncological prognosis, the short-term outcome of treatments, such as surgery or neoadjuvant chemoradiotherapy, and the future development of CRC. Developing nomograms always presents a dilemma. On the one hand, the desire to cover as wide a patient range as possible tends to produce nomograms that are too complex and yet have C-indexes that are not sufficiently high. Conversely, confining the target patients might impair the clinical applicability of constructed nomograms.

CONCLUSION: The information provided in this review should be of use in selecting a nomogram suitable for obtaining desired predictions in daily clinical practice.

Key words: Colon; Rectum; Nomograms; Prognosis; Cancer

Core tip: In this review, we discuss currently available colorectal cancer (CRC)-associated nomograms, including those that predict the oncological prognosis, the short-term outcome of treatments, such as surgery or neoadjuvant chemoradiotherapy, and the future development of CRC. This review aims to assist in the selection of suitable nomograms for obtaining desired predictions in daily clinical practice.

Kawai K, Sunami E, Yamaguchi H, Ishihara S, Kazama S, Nozawa H, Hata K, Kiyomatsu T, Tanaka J, Tanaka T, Nishikawa T, Kitayama J, Watanabe T. Nomograms for colorectal cancer: A systematic review. *World J Gastroenterol* 2015; 21(41): 11877-11886 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i41/11877.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i41.11877

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in Asia as well as in most Western countries[1]. A number of studies have suggested scoring or stratifying the risk associated with CRC, as represented by the American Joint Committee on Cancer TNM classifications[2-4]. A nomogram is a graphic calculating scale designed to provide the likelihood of the occurrence of a specific event. In clinical practice, a nomogram is typically used to predict the probability of a particular outcome as related to a disease. The clinical use of nomograms extends as far back as 1928 when nomograms were first used by Lawrence Henderson[5]. In recent years, a number of nomograms concerning the treatment of cancers, including prostate cancers[6], gastric cancers[7], and CRCs, have been reported because of their user friendly interface and strong statistical ability to predict individualized outcome.

In this systematic review, we discuss the currently available CRC-related nomograms, including those that predict the prognosis, the short-term outcome of treatments, such as surgery or neoadjuvant chem-oradiotherapy (CRT), and CRC prevalence.

MATERIALS AND METHODS

Evidence acquisition

We used PubMed to perform electronic searches for publications on CRC-associated nomograms. Our search included all English language entries from inception until February 2015 and incorporated the following keywords: nomogram/colon/rectal/colorectal in all fields. Only human studies were eligible for inclusion; case reports, editorials, letters, commentaries, and nomograms that were not published in print were excluded. Studies that only validated previously published nomograms without describing the development of new nomograms were also excluded. The initial search resulted in 174 publications. After title and abstract screening, 41 studies remained, and 28 were finally selected for the present review after full text screening (Figure 1).

RESULTS

Assessment of the predictive quality of nomograms

Before applying published nomograms to clinical practice, understanding the reliability of the predictions as well as the limitations of each nomogram is essential. First, the targeted patient characteristics and predicted outcomes should be noted. The targeted cancer location and the TNM stage varies among nomograms, and inputting data into a nomogram that was not developed pursuant to a particular patient's disease type might result in misreporting the probabilities. In Tables 1, 2, 3 and 4[8-42], we tabulated nomograms according to patient backgrounds for which the nomograms were developed as well as the intended outcomes with the aim of assisting clinical doctors in selecting the appropriate nomogram for their particular needs.

Second, the concordance index (C-index) is important. The C-index represents the ability of a model to reliably predict whether individuals more likely to experience the intended result and is equivalent to the area under the receiver-operator characteristic curve if there are no censored cases. A value of 0.5 indicates no predictive discrimination, whereas a value of 1.0 indicates perfect separation of patients with different outcomes. C-indexes of most nomograms ranged from 0.7 to 0.8, and those below 0.7 were regarded to have a relatively low prediction ability. Third, whether the validation of the nomogram was disclosed or not is also essential. Because the outcome of a treatment varies substantially between institutions, results from a single institution tend to be

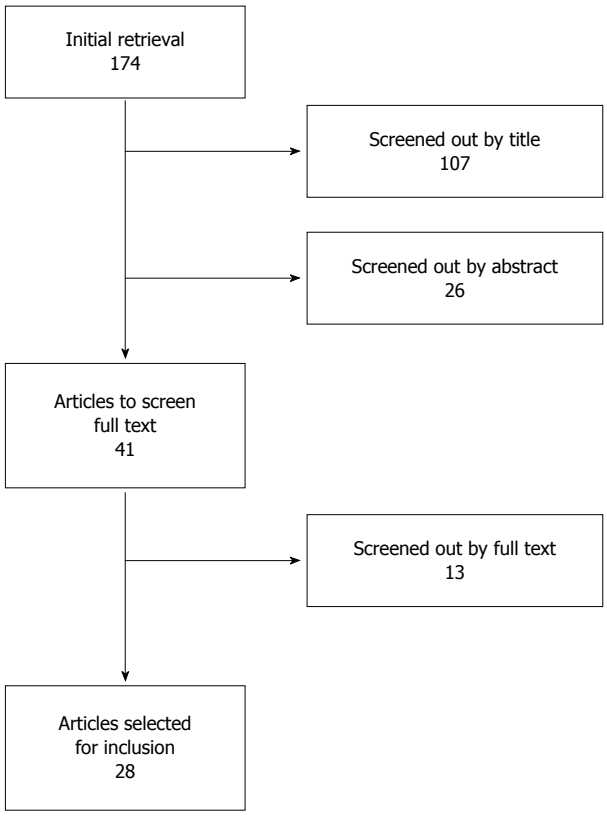

Figure 1 Flow chart of the study selection process.

Table 1 Nomograms predicting stage Ⅰ-Ⅲ colorectal cancer oncological prognosis

Ref.	Year	Cancer location	Targeted patients	Predicted outcome	Number of patients	C-index	Validation	Calibration	Variables	Comments
Weiser *et al*[8]	2008	Colon	Stage Ⅰ-Ⅲ	RFS	1320	0.77	Absent	Present	Age, CEA, No. of positive and negative nodes, pT, adjuvant chemotherapy, cancer location, differentiation, lymphovascular invasion, perineural invasion	
Segelman *et al*[9]	2014	Colorectal	Stage Ⅰ-Ⅲ	Peritoneal carcinomatosis	8044	0.78-0.80	Absent	Present	Age, cancer location, pT, pN, radicality, type of surgery, preoperative radiotherapy, nodes examined, adjuvant chemotherapy	Only web-calculator was available
Ying *et al*[10]	2014	Colorectal	Stage Ⅰ-Ⅲ	RFS, OS, CSS	205	0.80-0.81	Absent	Absent	Chemotherapy, tumor size, cell differentiation, TNM stage, neutrophil-to-lymphocyte ratio	
Zhang *et al*[11]	2013	Colon	Stage Ⅱ	RFS	735	0.65-0.82	Present	Present	Expression of microRNA, pT, internal obstruction or perforation, nodes examined, tumor grade	
Goossens-Beumer *et al*[12]	2015	Colorectal	Stage Ⅱ/Ⅲ	RFS	93	0.80	Present	Present	Expression of microRNA, TNM stage, age, gender	
Peng *et al*[13]	2014	Rectal	Stage Ⅱ/Ⅲ	OS, distant metastasis	883	0.68-0.76	Present	Absent	Gender, age, CEA, cancer location, pT, pN, ratio of metastatic lymph nodes, adjuvant chemotherapy, adjuvant chemoradiotherapy	
Valentini *et al*[14]	2011	Rectal	Clinical stage Ⅱ/Ⅲ patients undergoing adjuvant radiotherapy or chemora-diotherapy	OS, local recurrence, distant metastasis	2795	0.68-0.73	Present	Present	pT, cT, pN, age, concomitant and adjuvant chemotherapy, surgical procedure, gender, dose of radiotherapy	
van Gijn *et al*[15]	2015	Rectal	Stage Ⅰ-Ⅲ	OS, local recurrence, distant metastasis	2881	0.75-0.79	Absent	Absent	Age, pT, pN, PA-stage, distance, residual cancer, surgical type, gender, radiotherapy, complications	

RFS: Recurrence-free survival; OS: Overall survival; CSS: Cancer-specific survival; C-index: Concordance index; CEA: Carcinoembryonic antigen.

biased. If a reported C-index that used patient data from an external institution was comparable to the C-index of the derivation data set, the nomogram was regarded as generally applicable across institutions. Finally, a calibration plot should be provided. The C-index only provides the overall stratifying ability of a nomogram, whereas a calibration plot represents the actual correlation between the nomogram-predicted probability and the observed incidence.

Nomograms predicting stage Ⅰ-Ⅲ CRC oncological prognosis

In terms of nomograms that predict long-term prognosis after CRC surgery, no nomogram that predicts prognosis for all stages has been developed because the prognosis for stages Ⅰ-Ⅲ differs substantially from that of stage Ⅳ and variables associated with prognosis also differ markedly. As shown in Table 1, our search retrieved 8 nomograms predicting the prognosis of

Table 2 Nomograms predicting stage Ⅳ colorectal cancer oncological prognosis

Ref.	Year	Targeted cancer	Treatment	Predicted outcome	Number of patients	C-index	Validation	Calibration	Variables
Beppu *et al*[18]	2012	Liver metastasis	Hepatic resection	DFS	727	Not assessed	Validated by Okuno *et al*[26]	Absent	Metachronous or synchronous, pN, No. of tumors, largest tumor diameter, extrahepatic metastasis, CA19-9
Kanemitsu *et al*[19]	2008	Liver metastasis	Hepatic resection	OS, CSS	578	0.66-0.68	Validated by Takakura *et al*[27]	Present	Histology, No. of lymph node metastases, No. of tumors, extrahepatic metastasis, metastasis of hilar lymph nodes, surgical margin, CEA
Kattan *et al*[20]	2008	Liver metastasis	Hepatic resection	CSS	1477	0.61	Validated by Takakura *et al*[27], Reddy *et al*[28], and Nathan *et al*[29]	Present	Gender, age, primary site, disease-free interval, CEA, No. of tumors, largest tumor diameter, bilateral resection, > 1 lobe, pN
Kanemitsu *et al*[21]	2004	Lung metastasis	Thoracotomy	OS	313	0.66-0.72	Validated by Kanemitsu *et al*[30]	Present	Histology, No. of tumors, hilar/mediastinal lymph nodes, extrathoracic metastasis, CEA
Elias *et al*[22]	2014	Liver and/or Peritoneal metastasis	Optimal surgery plus chemotherapy	OS	287	0.61	Absent	Present	No. of lymph node metastases, peritoneal carcinomatosis index, planified procedure
Kawai *et al*[23]	2015	Metastatic CRC	Curative resection	DFS, OS	1133	0.60-0.64	Present	Present	Postoperative CEA, pT, pN, No. of metastatic organs, peritoneal dissemination
Manceau *et al*[24]	2014	Metastatic CRC, KRAS-wild-type, refractory to chemotherapy	Anti-EGFR antibodies	Risk of progression	132	> 0.7	Present	Absent	MicroRNA expression and BRAF mutations
Massacesi *et al*[25]	2000	Locally advanced or metastatic CRC	Chemotherapy	OS	1057	Not assessed	Absent	Absent	Response to chemotherapy, No. of metastatic sites, CEA, performance status

DFS: Disease-free survival; OS: Overall survival; CSS: Cancer-specific survival; CRC: Colorectal cancer; C-index: Concordance index; CEA: Carcinoembryonic antigen; KRAS: Kirsten rat sarcoma viral oncogene homolog; EGFR: Epidermal growth factor receptor; BRAF: B-Raf proto-oncogene, serine/threonine kinase.

stage Ⅰ-Ⅲ CRC patients[8-15]. Two nomograms were for colon cancer, three were for colorectal cancer, and the remaining three were for rectal cancer. Most of these nomograms were published within the past few years.

In 2008, Weiser *et al*[8] developed a nomogram predicting recurrence after surgery using general clinicopathological variables. Although the C-index of this nomogram was sufficiently high, the overall survival (OS) was not included in the outcome, and external validation was not performed. Recently, two nomograms for CRC, which were available in municipal hospitals, were published. One nomogram, developed by Segelman *et al*[9] was unique because it specialized in predicting peritoneal carcinomatosis recurrence. The other nomogram, developed by Ying *et al*[10], succeeded in achieving a high (greater than 0.8) C-index by adding preoperative neutrophil-to-lymphocyte ratio (NLR) to the conventional clinicopathological variables as an additional predictor. In several precedent studies, high NLR has been reported to correlate with a poorer prognosis in CRC[16,17], and this group established the clinical applicability of NLR by incorporating it into nomograms that calculated the probabilities of recurrence free survival (RFS), OS, and cancer-specific survival (CSS). Because the number of patients included was relatively small and no validation was performed, future studies validating the nomograms developed by Ying *et al*[10] with larger amounts of external patient data would reinforce their results. MicroRNA classifiers were incorporated in the remaining two nomograms. One such nomogram developed by Zhang *et al*[11] demonstrated that six microRNAs (miR-21-5p, miR-20a-5p, miR-103a-3p, miR-106b-5p, miR-143-5p, and miR215) independently predict prognosis, and one nomogram developed by Goossens-Beumer *et*

Table 3 Nomograms predicting short-term outcomes of surgery for colorectal cancer

Ref.	Year	Cancer location	Targeted patients	Predicted outcome	Number of patients	C-index	Validation	Calibration	Variables	Comments
Kiran *et al*[31]	2013	Colorectal	All colorectal surgeries	30-d mortality	30900	0.89	Present	Present	Age, ASA, albumin, functional dependency, renal failure, emergency surgery, disseminated cancer	
Hedrick *et al*[32]	2013	Colorectal	All colorectal surgeries	Superficial SSI, deep incisional SSI, and combination thereof	18403	0.64-0.65	Absent	Present	Diabetes, smoking, disseminated cancer, BMI, open or laparoscopic surgery	
de Campos-Lobato *et al*[33]	2009	Small bowel/ colorectal	All colorectal surgeries	Organ space SSI	12373	0.65	Present	Present	Surgical site, smoking, ASA, wound class, diabetes, steroid use, prior surgery, radiotherapy, open or laparoscopic surgery, age, BMI, creatinine, albumin, gender, transfusion, operative time	
Frasson *et al*[34]	2014	Colon	All colorectal surgeries	Anastomotic leakage	3193	0.62-0.63	Absent	Absent	Oral anticoagulants, intraoperative complications, BMI, total protein, gender, No. of beds	Decision-tree diagram was also presented
Yao *et al*[35]	2014	Rectal	Laparoscopic anterior resection with intracorporeal rectal transection and double-stapling technique anastomosis	Anastomotic leakage	476	0.84	Internal validation	Absent	Cancer location, operative time, preservation of the left colic artery	
Russell *et al*[36]	2013	Rectal/ rectosigmoid	Stage Ⅰ-Ⅲ	Rate of margin positivity	85190	0.75	Absent	Present	Age, gender, ethnicity, cancer location, TNM stage, tumor size, tumor grade, insurance status, histology	

SSI: Surgical site infection; ASA: American Society of Anesthesiologists; BMI: Body mass index; C-index: Concordance index.

al[12] focused on two microRNAs (miR-25-3p and miR-339-5p). Although these studies demonstrated the importance of microRNAs in CRC prognosis, currently, it may be difficult to apply these nomograms at municipal hospitals.

Three nomograms for rectal cancer prognosis have been reported to date. Most notably, the nomograms by Valentini *et al*[14] were developed using data from five major European clinical trials. Because OS, local recurrence, and distant metastasis were all included in the predicted outcome and because both validation and calibration were presented, these nomograms should have high clinical applicability. However, their usage is limited to patients who underwent radiotherapy or chemoradiotherapy (CRT).

Therefore, of the nomograms predicting stage Ⅰ-Ⅲ CRC prognosis, the nomograms developed by Weiser and Valentini for colon and rectal cancer, respectively, appear to be the most promising for clinical practice because, in these nomograms, the number of patients enrolled was large, no variables that are unavailable in municipal hospitals were incorporated, and the developed nomograms were well calibrated.

Nomograms predicting Stage Ⅳ colorectal cancer oncological prognosis

Nomograms predicting the prognosis of metastatic CRC are presented in Table 2[18-25]. Because stage

Table 4 Other nomograms relevant to colorectal cancer

Ref.	Year	Targeted patients	Treatment	Predicted outcome	Number of patients	C-index	Validation	Calibration	Variables	Comments
Jwa *et al*[37]	2014	Non-metastatic rectal cancer	CRT + surgery	ypN status	891	0.77-0.81	Present	Present	ypT, cN, histology, lymphovascular invasion, perineural invasion, age	
van Stiphout *et al*[38]	2011	Rectal cancer	CRT + surgery	Pathologic complete response	953	Not assessed	Present	Present	tumor length, RI, SUV	Pre- and post-CRT PET-CTs were used to predict response
van Stiphout *et al*[39]	2014	Rectal cancer	CRT + surgery	Pathologic complete response	190	0.70-0.78	Present	Absent	Maximal diameter at day 15, RI, cN	Pre- and intra-CRT PET-CTs were used to predict response
Omata *et al*[40]	2011	Asymptomatic individuals		Colorectal neoplasms	1085	Not assessed	Absent	Absent	Quantitative fecal immunochemical test, gender, age, BMI	
Kawai *et al*[41]	2014	Colorectal cancer	Surgery	Postoperative development of metachronous colorectal neoplasms	309	0.71	Present	Present	Gender, age, No. of synchronous adenomas and colorectal cancers	
Wells *et al*[42]	2014	Age > 45		Colorectal cancer development	180630	0.68	Absent	Present	Age, ethnicity, smoking, alcoholic drinks, BMI, education, aspirin, estrogen, family history of CRC, NSAIDs, multivitamins, red meat intake, diabetes, physical activity	

CRT: Chemoradiotherapy; PET-CT: Positron emission tomography-computed tomography; RI: Response index; SUV: Standardized uptake value; CRC: Colorectal cancer; BMI: Body mass index; C-index: Concordance index; NSAID: Non-steroidal anti-inflammatory drug.

Ⅳ CRC includes a wide variety of clinical settings, the C-indexes were relatively low with most being below 0.70. In contrast, most C-indexes of the nomograms for stage Ⅰ-Ⅲ CRC were above 0.75, as shown in Table 1. In terms of patients who underwent complete resection of metastases, three nomograms predicting the prognosis after resection of liver metastasis with curative intent have been established[18-20]; the widespread applicability of these nomograms was demonstrated by external validation studies[26-29]. These nomograms include both synchronous and metachronous liver metastasis, and two of these nomograms incorporated the interval between primary CRC surgery and hepatic resection as a variable because the prognosis of metachronous liver metastasis was better than that of synchronous lesions. Kanemitsu *et al*[21] and Kattan *et al*[20] demonstrated carcinoembryonic antigen (CEA) to be a strong prognosis-predictive marker, whereas Beppu focused on CA19-9. Kanemitsu *et al*[21] also constructed a nomogram predicting OS after thoracotomy for lung metastasis from CRC[21], which they subsequently validated in a separate study[30]. Elias *et al*[22] reported a nomogram specifically for those with liver and/or peritoneal metastasis and for those that underwent surgery including hyperthermic intraperitoneal chemotherapy (HIPEC) with no macroscopically residual cancer[22]. The nomogram was unique in that it was based on the outcome of 156 HIPEC patients. Recently, we built nomograms predicting DFS and OS after curative resection of stage Ⅳ CRC, namely, the complete resection of both primary CRC and synchronous distant metastasis[23]. We focused on the CEA concentration shortly after surgery because high postoperative CEA may be indicative of residual cancer cells and, consequently, of recurrence. The nomograms should have an advantage over previous nomograms because they may apply to all stage Ⅳ cases regardless of the metastatic organ, although their C-indexes were no

greater than 0.7, which is similar to other stage Ⅳ nomograms.

The remaining two nomograms predicted the outcome of chemotherapy for those who were unable to undergo complete surgical resection. One nomogram demonstrated the significance of hsa-miR-31-3p expression as a risk factor for cancer progression in patients who were refractory to chemotherapy and were treated with anti-EGFR therapy[24], and the other nomogram demonstrated the 2-year survival of locally advanced or metastatic CRC patients[25]. Because the latter was developed using patient data gathered between 1990 and 1998, the predicted survival may currently be improved due to the subsequent development of diverse chemotherapeutic agents.

Nomograms predicting short-term outcomes of surgery for colorectal cancer

There have been six published nomograms predicting short-term operative outcomes, namely, mortality[31], surgical site infection (SSI)[32,33], anastomotic leakage[34,35], and the rate of margin positivity[36] (Table 3). Because of the low incidences of these outcomes, most of the nomograms were constructed using large national databases such as the American College of Surgeons' National Surgical Quality Improvement Program; consequently, the numbers of enrolled patients were greater than 10000 in five of these nomograms, which was an order of magnitude greater than the number of patients in the majority of the studies predicting long-term oncological prognosis.

The 30-d mortality risk, which was the most serious postoperative complication, was predicted by Kiran *et al*[31]'s nomogram. This nomogram achieved a C-index of 0.89 by focusing particularly on age. There have also been three nomograms for calculating the incidence of SSI or anastomotic leakage in general colorectal surgery[32-34]. Because the occurrence of these complications was largely affected by the surgical procedures, it may be difficult to accurately anticipate the complications in advance using statistical models. Therefore, the C-indexes of these nomograms were only 0.65 at most. Recently, Yao *et al*[35] reported another nomogram predicting anastomotic leakage. Although its C-index was high (0.84), this exclusive nomogram only covered patients who underwent laparoscopic anterior resection with intracorporeal rectal transection and anastomosis using the double-stapling technique. In addition to postoperative complications, the rate of margin positivity in rectal cancer surgery was also predicted. Because the circumferential resection margin is a major determinant of local recurrence, predicting the rate preoperatively should be of considerable clinical benefit. However, a nomogram developed by Russell *et al*[36] incorporated factors that could not be confirmed preoperatively, such as tumor stage and size, and its actual clinical applicability was therefore limited.

Other nomograms relevant to CRC

Among the remaining six nomograms related to CRC, three concerned the prediction of the response to preoperative CRT in rectal cancer[37-39]. This was quite important in deciding the post-CRT treatment because accurate prediction of lymph node metastasis after CRT might enable the reduction of the surgical resection to local excision of the tumor instead of performing total mesorectal excision. Similarly, perfect prediction of the pathological complete response (pCR) might make it possible to omit even the surgery itself. One nomogram reported by Jwa *et al*[37] predicted the lymph node metastasis status of rectal cancer after CRT. Because this nomogram used the ypT stage, lymphovascular invasion, and perineural invasion as variables, it could not determine a suitable surgical procedure in advance. Alternatively, to clinically utilize the nomogram, local excision and pathological examination must first be performed, and if the risk of nodal metastasis calculated by the final pathological findings is acceptably low, omission of further surgical treatment accompanied by lymph node dissection could be one of the therapeutic options. van Stiphout *et al*[38] reported two nomograms predicting pCR by using positron emission tomography (PET)-computer tomography (CT) as the predictor[38,39]. In their first study, PET-CT was performed before and after CRT, and they incorporated the response ratio calculated by the standardized uptake values of these two PET-CTs into their nomogram[38]. Alternatively, they performed PET-CT before and two weeks after the start of CRT in their latter nomogram and demonstrated that the response ratio between the two PET-CT scans (*i.e.*, early response to CRT) is also a promising predictive factor available in the nomogram[39]. In the future, the accumulation of these data may enable the identification of patients who can either avoid unnecessary overtreatment or who should receive additional chemotherapy or radiotherapy.

Finally, we describe three nomograms that attempt to detect or predict newly developed CRCs[40-42]. Omata *et al*[40] demonstrated the diagnostic performance of the quantitative fecal immunochemical test (QTFIT) for colorectal neoplasms in asymptomatic individuals, and the addition of sex, age, and body mass index to the nomograms could amplify the accuracy of QTFIT as a screening test. Recently, we developed a nomogram that could predict the development of metachronous colorectal neoplasms after surgical resection of primary CRC[41] because patients who previously had CRC are at a high risk for developing second primary adenoma or CRC. Wells *et al*[42] also provided a nomogram calculating the 10-year risk of CRC development. The latter two nomograms were of clinical utility in identifying those patients who should receive intensive colonoscopy screening.

DISCUSSION

In the field of prostate cancer, a number of nomograms predicting a wide variety of outcomes, such as cancer prognosis[43], diagnosis[44], and screening[45], have been developed and well validated. In contrast, nomograms for CRC fall behind nomograms for prostate cancer, with the targeted patients and performed validation studies being limited. Therefore, further developments and validations of novel nomograms for CRC are needed. Developing nomograms always presents a dilemma. On the one hand, the desire to cover as wide a patient range as possible tends to produce nomograms that are too complex and yet have C-indexes that are not sufficiently high. Conversely, confining the target patients might impair the clinical applicability of constructed nomograms. The information provided in this review should be of use in selecting a nomogram suitable for obtaining desired predictions in daily clinical practice.

COMMENTS

Background

A nomogram is a graphic calculating scale designed to provide the likelihood of the occurrence of a specific event. In clinical practice, a nomogram is typically used to predict the probability of a particular outcome as related to a disease.

Research frontiers

In recent years, a number of nomograms concerning the treatment of cancers, including prostate cancers, gastric cancers, and colorectal cancers (CRCs), have been reported because of their user friendly interface and strong statistical ability to predict individualized outcome.

Applications

In this systematic review, the authors discuss the currently available CRC-related nomograms, including those that predict the prognosis, the short-term outcome of treatments, such as surgery or neoadjuvant chemoradiotherapy, and CRC prevalence. The information provided in this review should be of use in selecting a nomogram suitable for obtaining desired predictions in daily clinical practice.

Peer-review

It is an interesting paper with a good review of a frequently disperse information, well-written review and may have a potential significance for clinical practice of CRC.

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