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**OPINION REVIEW**

- 1847** Malignant gastric outlet obstruction: Which is the best therapeutic option?
Troncone E, Fugazza A, Cappello A, Del Vecchio Blanco G, Monteleone G, Repici A, Teoh AYB, Anderloni A

REVIEW

- 1861** Macrophages in metabolic associated fatty liver disease
Alharthi J, Latchoumanin O, George J, Eslam M

MINIREVIEWS

- 1879** Regulation of macrophage activation in the liver after acute injury: Role of the fibrinolytic system
Roth K, Strickland J, Copple BL
- 1888** Sequencing of systemic treatment for hepatocellular carcinoma: Second line competitors
Piñero F, Silva M, Iavarone M
- 1901** Therapeutic advances in non-alcoholic fatty liver disease: A microbiota-centered view
Chen HT, Huang HL, Li YQ, Xu HM, Zhou YJ

ORIGINAL ARTICLE**Basic Study**

- 1912** Interleukin-6 compared to the other Th17/Treg related cytokines in inflammatory bowel disease and colorectal cancer
Velikova TV, Miteva L, Stanilov N, Spassova Z, Stanilova SA
- 1926** Mutation analysis of related genes in hamartoma polyp tissue of Peutz-Jeghers syndrome
Zhang Z, Duan FX, Gu GL, Yu PF

Retrospective Study

- 1938** Iron metabolism imbalance at the time of listing increases overall and infectious mortality after liver transplantation
Fallet E, Rayar M, Landrieux A, Camus C, Houssel-Debry P, Jezequel C, Legros L, Uguen T, Ropert-Bouchet M, Boudjema K, Guyader D, Bardou-Jacquet E

Observational Study

- 1950** Effectiveness of very low-volume preparation for colonoscopy: A prospective, multicenter observational study
Maida M, Sinagra E, Morreale GC, Sferrazza S, Scalisi G, Schillaci D, Ventimiglia M, Macaluso FS, Vettori G, Conoscenti G, Di Bartolo C, Garufi S, Catarella D, Manganaro M, Virgilio CM, Camilleri S

Randomized Clinical Trial

- 1962** Retrograde inspection *vs* standard forward view for the detection of colorectal adenomas during colonoscopy: A back-to-back randomized clinical trial
Rath T, Pfeifer L, Neufert C, Kremer A, Leppkes M, Hoffman A, Neurath MF, Zopf S

CASE REPORT

- 1971** Severe steroid refractory gastritis induced by Nivolumab: A case report
Vindum HH, Agnholt JS, Nielsen AWM, Nielsen MB, Schmidt H
- 1979** Efficacy of bevacizumab-containing chemotherapy in metastatic colorectal cancer and CXCL5 expression: Six case reports
Novillo A, Gaibar M, Romero-Lorca A, Gilsanz MF, Beltrán L, Galán M, Antón B, Malón D, Moreno A, Fernández-Santander A

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Malignant gastric outlet obstruction: Which is the best therapeutic option?

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Abstract

Malignant gastric outlet obstruction (MGOO) is a clinical condition characterized by the mechanical obstruction of the pylorus or the duodenum due to tumor compression/infiltration, with consequent reduction or impossibility of an adequate oral intake. MGOO is mainly secondary to advanced pancreatic or gastric cancers, and significantly impacts on patients' survival and quality of life. Patients suffering from this condition often present with intractable vomiting and severe malnutrition, which further compromise therapeutic chances. Currently, palliative strategies are based primarily on surgical gastrojejunostomy and endoscopic enteral stenting with self-expanding metal stents. Several studies have shown that surgical approach has the advantage of a more durable relief of symptoms and the need of fewer re-interventions, at the cost of higher procedure-related risks and longer hospital stay. On the other hand, enteral stenting provides rapid clinical improvement, but have the limit of higher stent dysfunction rate due to tumor ingrowth and a subsequent need of frequent re-interventions. Recently, a third way has come from interventional endoscopic ultrasound, through the development of endoscopic ultrasound-guided gastroenterostomy technique with lumen-apposing metal stent. This new technique may ideally encompass the minimal invasiveness of an endoscopic procedure and the long-lasting effect of the surgical gastrojejunostomy, and brought encouraging results so far, even if prospective comparative trial are still lacking. In this Review, we described technical aspects and clinical outcomes of

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the above-cited therapeutic approaches, and discussed the open questions about the current management of MGOO.

Key words: Gastrojejunostomy; Self-expanding metal stent; Enteral stent; Interventional endoscopic ultrasonography; Endoscopic ultrasound-guided gastroenterostomy; Pancreatic cancer; Gastric cancer; Duodenal stricture

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Core tip: In the last decades, surgical gastrojejunostomy and enteral stenting have represented the main palliative strategies for patient with malignant gastric outlet obstruction. Although they showed good clinical efficacy, these approaches suffer from limits secondary to the high procedure-related risks and longer hospital stay (surgery) or the need subsequent re-interventions due to stent dysfunction (endoscopic stenting). The recently proposed endoscopic ultrasonography-guided gastroenterostomy may include both advantages of a minimally invasive endoscopic procedure and the long-lasting benefits of the gastrojejunostomy. However, such procedure is not standardized and prospective comparative studies are needed to define the best strategy for these patients.

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INTRODUCTION

Malignancies of the pancreas, biliary tract and gastro-duodenum are often diagnosed in advanced stages, in many cases are not amenable of curative surgical treatment and thus may require prolonged radio-chemotherapy regimens or palliative care. In this setting, malignant gastric outlet obstruction (MGOO) is defined as the mechanical obstruction of the pylorus or the duodenum secondary to compression/infiltration from advanced loco-regional malignancies that make difficult or even impossible the oral feeding (Figure 1). Patients with MGOO typically present with nausea and vomiting, which could associate with abdominal pain, weight loss, malnutrition and dehydration secondary to poor oral intake^[1]. The most frequent cause of MGOO in the Western countries is pancreatic adenocarcinoma, which lead to obstructive symptoms in about 15%-20% of patients during the disease course, while gastric adenocarcinoma is the most common cause in Asiatic population^[2-7]. Less common causes are duodenal or ampullary neoplasms, biliary cancers, lymphomas or adenopathies from other malignancies^[1]. Effective treatment of MGOO is of paramount importance either for patients who have to face radio-chemotherapy regimens, as for those at late stage of disease who only require supportive care aimed at improving quality of life. For many decades MGOO has been managed with open surgical gastrojejunostomy, during which also biliary bypass was performed in case of concomitant biliary obstruction^[8-10]. However, most patients with MGOO often present with advanced disease and are not optimal candidates for open surgery. Due to the high surgical risk and the short life expectation (*i.e.* 3-4 mo) that characterize the majority of these patients, less invasive approaches have been developed and proposed over time, aimed at providing fast and effective relieve of symptoms and return to an adequate oral feeding with the highest safety, the shortest hospitalization time and the lowest costs. Such an ambitious goal has been pursued with the use of enteral stents, with the development of less invasive surgical techniques (*i.e.* laparoscopic gastrojejunostomy) and, more recently, with the progress of inter-ventional endoscopic ultrasonography (EUS), and the development of EUS-guided gastroenterostomy techniques (EUS-GE) (Figure 2). A consistent body of literature exists about the outcome and safety of such interventions in the setting of MGOO. Nevertheless, few randomized clinical trials have been conducted to compare these different approaches, and so there is still uncertainty about the best strategy to choose.

In this review, we aimed to summarize the available evidence on the most common palliative strategies for MGOO, focusing on the strength and weakness of each

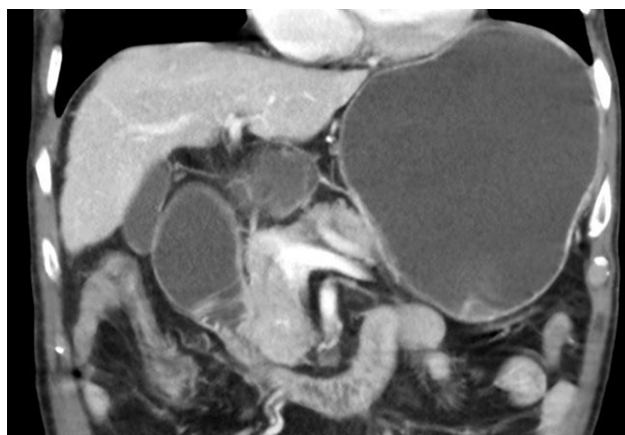


Figure 1 Computed tomography scan appearance of malignant duodenal stricture with gastric distension due to pancreatic cancer.

approaches and discussing possible unanswered questions that should be addressed in future studies.

ROLE OF ENTERAL STENTS

From the late 90's endoscopic enteral stenting has been proposed as a minimal invasive treatment for MGOO, using the experience gained from the use of expanding and self-expanding metal stents (SEMS) in the setting of malignant esophageal strictures^[11-13]. Generally, a wire is passed through the gastro-duodenal stricture under endoscopic and fluoroscopic assistance, and subsequently the metal stent is passed over the wire and released across the stenosis. According to specific endoscopist's preference, a soft angled wire can be used first to pass the stricture, and then exchanged with a stiffer one using a catheter. Moreover, passing a catheter over the wire allow to inject contrast to define the anatomy (*i.e.* length, angulation) of the stenosis, in order to optimize the size of the stent. Over the time, different techniques to deploy enteral stents across gastro-duodenal strictures have been described. After positioning a wire across the stricture, interventional radiologists could deploy the stent with an over-the-wire technique exclusively under fluoroscopic assistance^[14,15]. Alternatively, the stent can be deployed under endoscopic and fluoroscopic view; using the over-the-wire technique the endoscope is positioned parallel to the wire, while, with the through-the scope technique, the stent is inserted over the wire into the working channel of the endoscope^[16-19]. Currently, the endoscopic deployment using through-the scope stents is the most used technique, and requires therapeutic endoscopes with a large working channel (*i.e.*, ≥ 3.7 mm). Most cases are managed with therapeutic gastroscopes, but cases of dilated stomachs or strictures in the distal duodenum could be better managed with a colonoscope or a duodenoscope^[20,21]. Moreover, tight and angulated stenosis could be negotiated in an easier way using a sphincterotome, with the additional advantage of the elevator of the duodenoscope. The choice of the stent largely depend on the stricture anatomy and the endoscopist's preference. Over time, several different types of enteral metal stent have been designed, which differed in type of metal alloy, length, diameter and radial expansive force after deployment^[11,18,22-25] (Figure 3). Currently, available enteral SEMS are made of nitinol, an alloy of nickel and titanium, which confers high flexibility useful for sharply angulated strictures, even if with a weaker expansive radial force compared to other metal stents^[26]. Several studies on enteral SEMS for MGOO have shown a high rate of technical success (defined as the successful deployment of the stent across the stricture), which is usually above 90%, and a good rate of clinical success, which ranges from 63% to 97%^[27]. Clinical success is generally defined as the relief of obstructive symptoms and the improvement of food oral intake. Adler and colleagues developed a clinical score aimed at providing an objective measure of the oral intake before and after the treatment for MGOO^[19]. The Gastric Outlet Obstruction Scoring System (GOOSS) assigns a score of 0 in case of no oral intake, 1 for only liquids, 2 for soft solids and 3 for low-residues or full diet, and currently is the most used score to quantify the clinical improvement after treatment for MGOO^[19]. However, high heterogeneity exists among published studies on the definition of this outcome, and a systematic review from Larssen *et al*^[28] highlighted that only 40% of studies used a

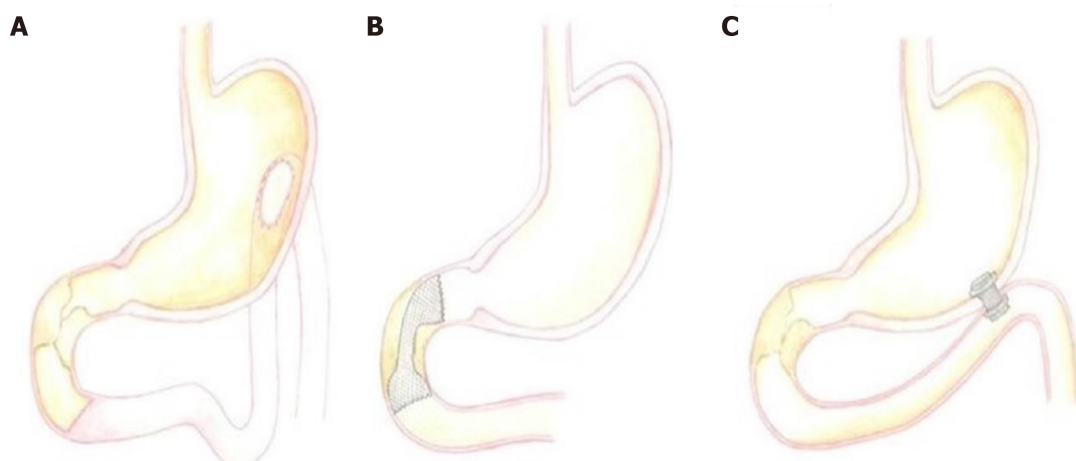


Figure 2 Graphic representation of the main approaches applied to manage malignant gastric outlet obstruction. A: Surgical gastroduodenostomy; B: Endoscopic enteral stenting with self-expanding metal stents; C: Endoscopic ultrasound-guided gastroenterostomy.

graded scoring system to evaluate the effect of stenting on MGOO. A systematic review of 32 studies (606 patients) reported a technical success rate of 97% and a clinical success rate of 89% (87% in the intention-to-treat analysis), with a mean GOOSS that rose from 0.4 to 2.4 after treatment, and a resolution of symptoms after a mean period of 4 d^[29]. Similarly, a systematic review of 1046 patients treated with duodenal SEMS published in 2007 reported a technical success and clinical success rate of 96% and 89% respectively, with a significant improvement of GOOSS^[30]. A recent systematic review from van Halsema and colleagues included 19 prospective studies from 2009 to 2016 and analyzed outcomes of more than 1200 patients with MGOO treated with SEMS. The overall pooled technical success rate was 97.3% and the clinical success rate was 85.7%, thus confirming the high efficacy of this technique^[27]. Several studies investigated potential predictive factors of clinical failure or stent dysfunction, in order to optimize the outcome of patients with MGOO undergoing stent placement. The presence of carcinomatosis and a poor performance status (Karnofsky performance status < 50 or Eastern Cooperative Oncology Group status ≥ 3) have been identified as predictors of clinical failure and/or stent dysfunction in several studies^[31-34], while chemotherapy after stent placement has been reported as protective^[35,36]. In particular, a retrospective study of 228 patients found that carcinomatosis is a predictive factor of clinical failure only if associated with ascites, while carcinomatosis without ascites did not decrease clinical success rate compared to patients without peritoneal disease^[37]. The site of the gastro-duodenal stricture (distal *vs* proximal) and the number of strictures (*i.e.*, ≥ 3) are other factors associated with worst outcome in retrospective studies^[31,38].

Adverse event (AE) rate related to SEMS placement ranges from 0% to 30% depending on the definition adopted in the specific study, and includes minor AEs (non-life threatening) such as nausea, vomiting, mild abdominal pain, or major AE, such as bleeding, perforation, stent migration/displacement, cholangitis^[19,29,30]. Delayed AEs are usually related to stent dysfunction, secondary to migration or occlusion by food impaction and/or tumor ingrowth/overgrowth. In the report of 1281 patients that received duodenal SEMS, stent obstruction was reported in 12.6%, stent migration in 4.3%, bleeding in 4.1% (major bleeding in 0.8%) and perforation in 1.2%^[27]. A recent retrospective study of 220 patients reported a SEMS-related AE rate of 2%, with 3 fatal cases of perforation and an overall rate of re-intervention of 13% after 4 mo^[39]. As stated above, stent may occlude due to food impaction or secondary to tumor progression and ingrowth, that is the tumor growth through the mesh of the stent. Stent occlusion leads to reappearance of gastric obstruction symptoms and often needs endoscopic re-intervention that, although feasible and effective, may affect negatively patients' quality of life and increase costs for the health system, representing one of the main weakness of this approach^[40,41]. In order to reduce the risk of stent occlusion, several studies have investigated the possible role of covered SEMS in the setting of MGOO. A systematic review and meta-analysis published in 2014 including 9 studies (849 patients) confirmed that covered SEMS have a lower obstruction rate (RR: 0.42, 95%CI: 0.24-0.73, $P = 0.002$), but at the same time, as expected, have a higher migration risk (RR: 3.48, 95%CI: 2.16-5.62, $P < 0.00001$)^[42]. Interestingly, the Authors reported no significant difference in technical success rate, clinical success rate, post-stenting dysphagia score, stent patency, overall com-

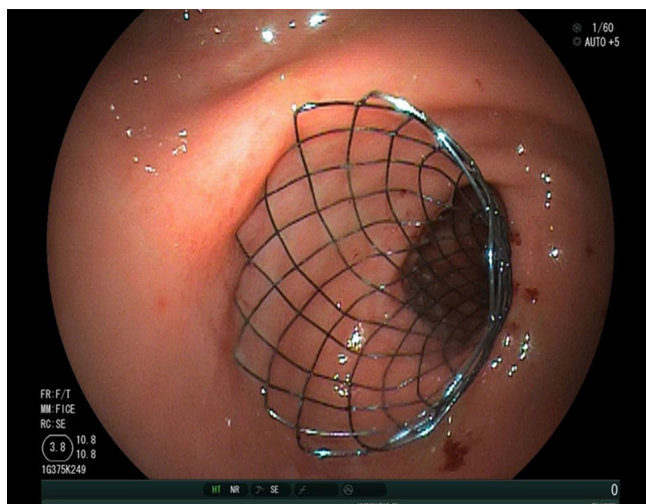


Figure 3 Final endoscopic appearance of a duodenal uncovered self-expanding metal stent deployed across duodenal stricture, in a patient with gastric outlet obstruction due to pancreatic cancer.

plications and re-intervention rate between covered and uncovered SEMS group. A more recent systematic review including 1624 patients reported similar results, with comparable technical and clinical success rate between covered and uncovered gastroduodenal SEMS^[43]. The Authors highlighted a trend toward a lower dysfunction rate in covered SEMS group (RR: 0.63; 95% CI: 0.45-0.88) when performing a sub-analysis of randomized trials. However, it should be noted that the higher risk of migration was confirmed for covered SEMS, together with a higher overall AE rate in this group (RR: 1.75; 95% CI: 1.09-2.83)^[43]. Several technical modifications or precautions (*e.g.*, stent clipping or suturing, anti-migratory design) have been proposed to overcome the migration risk saving the possible advantages of the lower occlusion rate^[44-46]. Despite these intriguing alternatives, an increased risk of cholangitis and pancreatitis secondary to the compression/occlusion of the ampulla exists with covered SEMS^[22,47-49], and, therefore, uncovered SEMS are still considered the first option in this setting.

Advanced gastro-duodenal or pancreato-biliary malignancies frequently cause biliary obstruction, which is estimated to affect 70%-90% of pancreatic cancer patients during the course of the disease and may appear before, concomitantly or after the onset of MGOO^[1]. The presence of MGOO could make the papilla not achievable for standard endoscopic drainage with endoscopic retrograde cholangio-pancreatography (ERCP), in particular for type 1 (proximal to the papilla) or type 2 (at the level of the papilla) duodenal stricture, accordingly to the classification proposed by Mutignani *et al.*^[50]. Such cases may be very difficult for therapeutic endoscopists and, therefore, performing biliary drainage with biliary SEMS in patients concomitantly treated for MGOO with risk of impending or future biliary obstruction appears a reasonable strategy, when feasible^[50,51]. Although technically challenging, ERCP through an indwelling duodenal stent is feasible and effective, as reported in a recent multicenter retrospective studies on 71 patients, with an overall technical success rate of 85%, which was reduced to 76% in case of duodenal obstruction at the level of the ampulla^[52]. The recent progress of interventional EUS, and the possibility to perform EUS-guided biliary drainage (EUS-BD) from the stomach (*i.e.*, EUS-guided hepato-gastrostomy) or from the bulb (EUS-guided choledochoduodenostomy) has radically changed the approach to the patients with concomitant MGOO and biliary obstruction^[53]. EUS-BD in patients with MGOO is safe and effective, even when performed in the same session or with an indwelling duodenal stent, and probably could be considered the first-line strategy to achieve biliary drainage in this setting of patients^[54-56].

COMPARISON BETWEEN ENTERAL STENTING AND SURGICAL GASTROJEJUNOSTOMY

Since the introduction of enteral SEMS for palliation of MGOO, a consistent body of literature has been produced to compare this approach to surgical gastrojejunostomy (GJ), aimed at defining which method, and for which patients, had to be preferred in

this clinical setting (Figure 4). Currently, several retrospective cohort studies, but few randomized controlled studies, are available on this topic, with some conflicting results. A multicenter, prospective, randomized study conducted in The Netherlands between 2006 and 2008 randomized 39 patients with MGOO to duodenal stent placement (21 patients) or surgical GJ (18 patients) (SUSTENT Study)^[57]. The Authors found a faster relief of symptoms and improvement of GOOSS in the stent group compared to the surgery group (GOOSS score ≥ 2 after a median period of 5 *vs* 8 d, respectively; $P < 0.01$), but a more lasting relief in the surgery group, which showed a median period of 72 *vs* 50 d ($P < 0.05$) with GOOSS score ≥ 2 after the procedure. Major AEs, recurrent obstructive symptoms and re-interventions were more common after stent placement compared with GJ, and this result was mainly dependent on the risk of stent obstruction in the duodenal stent group^[57]. The authors concluded that GJ was the treatment of choice in patients with a life expectancy of 2 mo or longer because of the better long-term results, while stent placement was preferable for patients expected to live less than 2 mo due to the better short-term outcomes. Other randomized trials reported favorable results for the duodenal stenting, mainly in terms of shorter hospitalization time compared to surgical GJ^[58,59] (Table 1). However, it should be noted that the cited studies suffer from several limitations, especially for the limited number of patients enrolled and the lack of an adequate statistical power calculation. A recent retrospective study from Jang and colleagues analyzed the outcome of 183 patients who underwent SEMS placement and 127 patients who received surgical GJ over period of 7 years^[60]. While the clinical success did not differ significantly between the two groups (79.4% *vs* 80.1%; $P = 0.83$), the mean patency duration was significantly longer in the GJ group compared to the stenting group (169.2 *vs* 96.5 d respectively). Moreover, the GJ group showed longer survival (193.4 *vs* 119.9 d), and the authors concluded that GJ should be considered the primary treatment option for patients with good performance status and reasonable survival expectancy^[60]. It should be underlined that the work from Jang *et al*^[60] suffers from possible selection bias, which is the main limitation of many retrospective studies in this field. Indeed, patients who underwent surgery were “healthier” compared to patients selected for endoscopic palliation (Eastern Cooperative Oncology Group score of 1 *vs* 2, respectively; $P < 0.001$), and this could account for the longer survival in this group. Despite the unquestionable value of the study, the presented results are certainly not conclusive, and high quality evidence is still lacking. Recently, results from 27 studies including 2,354 patients (1,306 treated with SEMS and 1,048 with surgical GJ) have been analyzed in a systematic review with meta-analysis, which concluded that patients with acceptable performance status should be primarily considered for a palliative GJ rather than duodenal stenting^[61]. In particular, the study confirmed that mean time to oral intake and length of hospital stay were shorter in the SEMS group compared to surgery group, while the frequency of re-interventions was almost three times higher in the SEMS group (OR: 2.95, CI: 1.70-5.14, $P < 0.001$), thus remarking that stent dysfunction secondary to migration/dislocation or occlusion/obstruction is the main limitation of the duodenal stenting in MGOO^[61]. Surgical techniques other than classic open surgical gastrojejunostomy or laparoscopic gastrojejunostomy have been proposed to overcome the risk of post-operative delayed gastric emptying, such as stomach-partitioning GJ with or without Braun enterenterostomy, and small retrospective studies have compared such technique with enteral stenting^[62,63]. Although intriguing, the study design and the limited number of patients included do not yet allow drawing conclusion about the superiority of this approach over classic surgery or stenting, and larger studies are needed. Comparison of costs between GJ and stenting has produced conflicting results, as the shorter hospitalization documented with enteral stents could be counter-balanced by higher costs for re-interventions^[4,57,61,64,65]. Taken together, these data highlight that a correct selection of patients is probably the crucial step to achieve a satisfactory clinical outcome for both strategies.

ENDOSCOPIC ULTRASONOGRAPHY-GUIDED GASTROENTEROSTOMY

The above-cited progress of interventional EUS have enriched the field of therapeutic endoscopy with the possibility to perform trans-luminal procedures, such as trans-gastric or trans-duodenal drainage of peripancreatic fluid collections, gallbladder drainage in patients unfit for surgery or biliary drainage after failed ERCP^[66-69]. In the last years, it has been developed and proposed an innovative technique that allows the creation of a stable gastro-jejunal anastomosis through a EUS-guided procedure, named EUS-guided gastroenterostomy (EUS-GE)^[70,71]. With this regard, the field of

Table 1 Characteristics of the available randomized trials comparing duodenal stenting and surgical gastrojejunostomy for malignant gastric outlet obstruction

Ref.	Randomized patients	Treated patients	Type of stent	Surgical technique	Main outcome measures	Technical success	Clinical success	Adverse events	Hospital stay (d, median)	Reintervention	Follow-up
Fiori <i>et al</i> ^[58] , 2004	Stent group: 9	9	Covered SEMS (Ultraflex, Boston)	Open GJ	Gastric emptying (after 15 d)	9/9 (100%)	9/9 (100%)	2/9 (22.2%)	3.1	7/9 (77.7%)	3 mo
	Surgery group: 9	9				9/9 (100%)	8/9 (88.9%)	2/9 (22.2%)	10	1/9 (11.1%)	
Mehta <i>et al</i> ^[59] , 2006	Stent group: 13	12	Wallstent (Boston) ¹	Laparoscopic GJ	Safety quality of life	10/12 (83.3%)	Significant improvement in Physical Health score after 1 mo in duodenal stent group	0/10 (0%)	5.2	NA	12 mo
	Surgery group: 14	13				13/13 (100%)		10/13 (76.9%)	11.4		
Jeurnink <i>et al</i> ^[57] , 2010	Stent group: 21	20	Wallflex (Boston)	Laparoscopic or open GJ	GOOSS improvement	20/21 (95.2%)	17/21 (80.9%)	8/21 (38.1%)	7	2/21 (9.5%)	Median survival: 72 d (GJ) vs 50 d (SEMS)
	Surgery group: 18	17				17/18 (94.4%)	14/18 (77.7%)	5/21 (23.8%)	15	7/18 ² (38.8%)	

¹In this study, the stents were positioned under fluoroscopic guidance.

²Seven patients experienced 10 adverse events. SEMS: Self-expanding metal stent; GJ: Gastrojejunostomy; GOOSS: Gastric outlet obstruction scoring system.

interventional has enormously benefited from the development of dedicated metal stents for trans-luminal interventions. Indeed, lumen-apposing metal stents (LAMS) are fully covered “dumb-bell”-shaped short stent made up of braided nitinol, specifically designed for interventional EUS procedures, with wide anti-migratory flanges which provide a lumen-to-lumen apposition effect^[68]. The stent is pre-loaded in a 10.8 French catheter with a through-the-scope delivery system compatible with therapeutic echoendoscope with a working channel ≥ 3.7 mm. In the EUS-GE procedure, the small bowel is punctured from the stomach at the level of the distal duodenum or in the proximal jejunum (*i.e.* gastroduodenostomy or gastrojejunostomy, respectively) under EUS and fluoroscopic guidance, with subsequent placement of a LAMS, thus creating a tight and sealed anastomosis, owing to the lumen-to-lumen apposition effect of the stent. The first EUS-GE with LAMS (AXIOS™ stent; Boston Scientific, Marlborough, MA, United States) was described in 2012 in a porcine model by Binmoeller and Shah^[72]. In the subsequent years, three different techniques have been described to perform EUS-GE with LAMS: (1) Direct EUS-GE; (2) Assisted EUS-GE, performed using accessory devices (*e.g.*, dilating balloon, single balloon overtube, nasobiliary drain, ultra-slim endoscope) for small bowel loop distension before puncture and stent placement; and (3) EUS-guided double balloon-occluded gastrojejunostomy bypass (EPASS)^[70,73-75].

Direct EUS-GE requires as first step the puncture of the small bowel loop from the stomach with a 19 Gauge fine needle aspiration (FNA) needle under EUS view. Once confirmed the positioning of the FNA needle into the small bowel through contrast injection under fluoroscopic view, a guide-wire is advanced through the needle into the small bowel, and dilation of the tract with a balloon or a cystotome is performed, to allow the insertion of the stent delivery system (10.8 French) into the small bowel and to open the LAMS. The main technical issues of this technique concern the correct puncturing of the small bowel loop, which is often collapsed and mobile, and the multi-step technique, with several subsequent device exchanges that increase the risk of pushing away the bowel loop with the guide-wire with possible subsequent leakage, perforation or stent mal-deployment. To address these limitations, injection of normal saline (usually about 500 mL) through the duodenal stricture and administering anti-peristaltic drug (*e.g.* glucagon) could help to reduce peristalsis and to have a good view of the target bowel loop. Moreover, LAMS delivery system has further evolved with the addition of an electrocautery tip [electrocautery-enhanced (EC)-LAMS-HOT-AXIOS™, Boston Scientific Corp., Marlborough, Massachusetts, United States] which allows a single-stage access to the small bowel distal to the obstruction, without the need of multiple exchanges^[67,76-78].

As resumed above, the assisted EUS-GE technique requires the distension of the

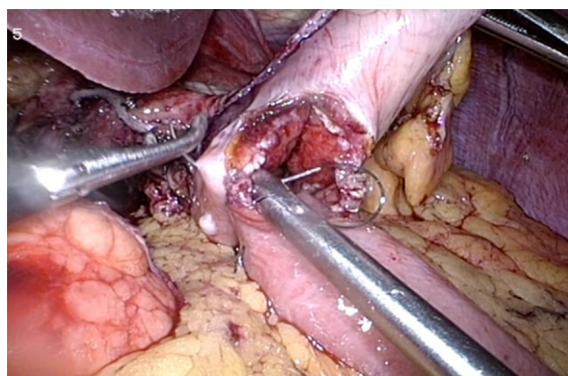


Figure 4 Intra-operative image of laparoscopic gastrojejunostomy.

jejunal loops distal to the strictures with infusion of normal saline directly injected through an ultra-slim endoscope which is advanced (when possible) beyond the stenosis or passing a nasobiliary catheter over a wire. Alternatively, a balloon is passed over a wire into the jejunum and then inflated, providing a guide for the EUS view to identify the target bowel loop (Figure 5). The main limitation of this technique is related to the difficulties in advancing per-orally the mentioned devices through an often tight and angulated duodenal stricture.

The EPASS technique requires a special double-balloon enteric tube (Tokyo Medical University type; Create Medic Co., Ltd, Yokohama, Japan) specifically designed for this procedure^[79,80]. The double-balloon tube is inserted perorally over a previously placed guidewire and advanced through the stenosis. Then, both balloons are filled with saline to hold the small intestine open and fixed, and saline with contrast material is introduced into the space between the two balloons to distend the small bowel lumen (Figure 6). At this point, the echoendoscope is introduced into the stomach and the distended duodenum or jejunum is identified at the EUS image. The subsequent LAMS placement can be performed with the multi-step procedure described above, or with a single-step procedure using the EC-LAMS.

Regardless of the technique adopted, data from several studies reported high technical and clinical success rate for EUS-GE in MGOO using 10 mm or 15 mm diameter LAMS, ranging from 87%-96% and 81%-92% respectively^[81]. In 2015, Khashab *et al*^[70] and colleagues reported the first series of EUS-GE in both malignant (3 patients) and benign (7 patients) gastric outlet obstruction using the direct or the balloon-assisted technique^[70]. The authors reported a technical success of 90% and clinical success of 100%, with resumption of soft or normal diet in all patients with technical success. Moreover, no AEs were reported and patients did not experienced symptom recurrence during a mean follow-up period of 150 d^[70]. Itoi *et al*^[82] reported similar outcomes in a prospective study of 20 EUS-GE performed with the EPASS technique, with a technical success of 90% and a significant improvement of GOOSS. Despite a 10% (2 patients) with stent mal-positioning, no further AEs were reported and no patients reported stent migration or occlusion needing re-interventions^[82]. Other series reported a rate of AEs ranging from 0 to 21%, including pneumoperitoneum, gastric leak, bleeding, peritonitis or abdominal pain^[74,80,83-85]. A multicenter study comparing the direct and the balloon-assisted technique reported no significant differences in technical and clinical success and AE rate^[86]. EUS-GE has been compared to enteral stenting in two retrospective studies^[80,85]. Technical success, length of hospitalization and safety were similar, while in the study from Ge *et al*^[85] a higher rate of initial clinical success was found in the EUS-GE group (95.8% *vs* 76.3%, $P = 0.042$)^[85]. Strikingly, stent failure requiring re-intervention was significantly lower in EUS-GE group compared to enteral stent group in both studies. A multicenter retrospective study compared EUS-GE (30 patients) and surgical GJ (63 patients)^[83]. Despite surgical GJ showed a higher technical success (100% *vs* 87%, $P = 0.009$), clinical success and symptoms recurrence were similar, even if a trend toward more frequent recurrent obstruction in the EUS-GE group (3% *vs* 14%, $P = 0.08$) was reported. A non-significant higher rate of AE rate was found in surgical GJ group (16% *vs* 25 %, $P = 0.3$), however it should be noted that surgery group underwent open GJ, and therefore these results may be not generalizable to laparoscopic GJ^[83] (Table 2).

Taken together, these data propose EUS-GE as a valuable minimal invasive option for patient with MGOO (Figure 7). The main limitations concern the technical difficulty of the procedure, which is not yet standardized and requires high skilled



Figure 5 Endoscopic ultrasound view of the distended jejunal loop.

therapeutic endoscopists. Moreover, high quality prospective studies comparing the three different palliative strategies are still lacking.

CONCLUSION

Palliation of MGOO may be challenging, and multi-disciplinary team is often needed to evaluate the best therapeutic strategy taking into account the patient's performance status, life expectancy, the need of chemo-radiotherapy, surgical risks and, importantly, the patient's preference. In this scenario, the therapeutic endoscopist may offer effective minimal invasive approaches. Enteral stenting provides rapid relief of obstructive symptoms and a short hospital stays through a relatively safe endoscopic procedure compared to surgical GJ. On the other hand, SEMS suffer from high rate of stent failure and need of re-intervention on long-term period, mainly secondary to stent ingrowth, and, for this reason, are the first-line strategy in ill patients with short life expectancy (< 3 mo). The recently proposed EUS-GE has the ambition to provide a minimal invasive endoscopic procedure, with the consequent safe and rapid efficacy, and, at the same time, with the long-lasting advantages of GJ, as the metal stent is placed away from the neoplastic stricture and therefore is virtually free from ingrowth risk. Despite these exciting novelties, EUS-GE is still a difficult and not standardized technique, and is currently limited to centers with high experience in therapeutic EUS. In the next years, conducting well-designed prospective studies will be the intriguing challenge to identify the best therapeutic option to treat patients with MGOO.

Table 2 Characteristics of the studies comparing endoscopic ultrasound-guided gastroenterostomy with surgical gastrojejunostomy or duodenal stenting in gastric outlet obstruction

Ref.	Study characteristics	EUS-GE technique	Comparison group	Number of patients	Technical success	Clinical success	Hospital stay (d, median)	Symptom recurrence or re-intervention	Adverse events
Chen <i>et al</i> ^[80] , 2017	Multicenter; Retrospective	EPASS; Balloon-assisted; Direct	Duodenal SEMS	EUS-GE: 30; SEMS: 52	26/30 (86.7%); 49/52 (94.2%)	25/30 (83.3%); 35/52 (67.3%)	11.3 ± 6.6; 9.5 ± 8.3	1/30 (4.3%); 10/52 (28.6%)	5/30 (16.7%); 6/52 (11.5%)
Khashab <i>et al</i> ^[83] , 2017	Multicenter; Retrospective	EPASS; Balloon-assisted; Direct	Open GJ	EUS-GE: 30; Open GJ: 63	26/30 (87%); 63/63 (100%)	26/30 (87%); 57/63 (90%)	11.6 ± 6.6; 12 ± 8.2	1/30 (3%); 9/63 (14%)	5/30 (16.7%); 16/63 (25%)
Perez-Miranda <i>et al</i> ^[84] , 2017	Multicenter; Retrospective	Assisted; Direct	Laparoscopic GJ (LGJ)	EUS-GE: 25 ¹ LGJ: 29	23/25 (88%); 29/29 (100%)	21/25 (90%); 28/29 (90%)	9.4; 8.9	NA; NA	3/25 (12%); 12/29 (41%)
Ge <i>et al</i> ^[85] , 2019	Single-center; Retrospective	Assisted; Direct	Duodenal SEMS	EUS-GE: 22; SEMS: 78	22/22 (100%); 78/78 (100%)	21/22 (95.5%); 60/78 (76.3%)	7.4 ± 9.1; 7.9 ± 8.2	2/22 (8.3%); 31/78 (32%)	5/22 (20.8%); 39/78 (40.2%)

¹Eight patients out of 25 (8/25) underwent endoscopic ultrasonography-guided gastroenterostomy for benign obstruction. EUS-GE: Endoscopic ultrasonography-guided gastroenterostomy; EPASS: Endoscopic ultrasonography-guided double balloon-occluded gastrojejunostomy bypass; SEMS: Self-expanding metal stent; GJ: Gastrojejunostomy.

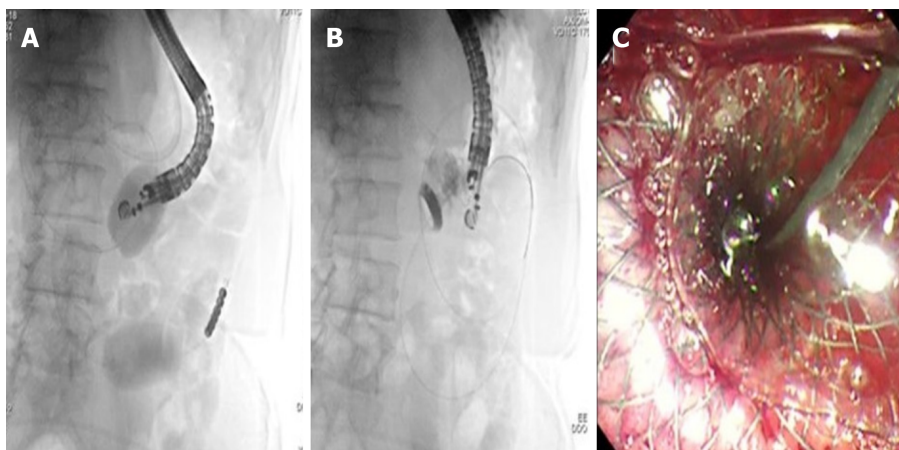


Figure 6 Endoscopic ultrasound-guided gastroenterostomy with the double balloon occluder. A: The double balloon occluder in place distending the small bowel in between; B: Endoscopic ultrasound-guided placement of lumen-apposing metal stent between the stomach and jejunum; C: Final endoscopic appearance of lumen-apposing metal stent.



Figure 7 Computed tomography scan appearance of endoscopic ultrasound-guided gastroenterostomy with lumen-apposing metal stent placed between the stomach and jejunum.

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Macrophages in metabolic associated fatty liver disease

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Abstract

Metabolic associated fatty liver disease (MAFLD), formerly named non-alcoholic fatty liver disease is the most common liver disorder in many countries. The inflammatory subtype termed steatohepatitis is a driver of disease progression to cirrhosis, hepatocellular carcinoma, liver transplantation, and death, but also to extrahepatic complications including cardiovascular disease, diabetes and chronic kidney disease. The plasticity of macrophages in response to various environmental cues and the fact that they can orchestrate cross talk between different cellular players during disease development and progression render them an ideal target for drug development. This report reviews recent advances in our understanding of macrophage biology during the entire spectrum of MAFLD including steatosis, inflammation, fibrosis, and hepatocellular carcinoma, as well as for the extra-hepatic manifestations of MAFLD. We discuss the underlying molecular mechanisms of macrophage activation and polarization as well as cross talk with other cell types such as hepatocytes, hepatic stellate cells, and adipose tissue. We conclude with a discussion on the potential translational implications and challenges for macrophage based therapeutics for MAFLD.

Key words: Metabolic associated fatty liver disease; Macrophages; Inflammation; Fibrosis; Hepatic stellate cells

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Core tip: In this work, we review the recent advances in our understanding of macrophage biology during the entire spectrum of metabolic associated fatty liver disease (MAFLD) including steatosis, inflammation, fibrosis, and hepatocellular carcinoma, as well as for the extra-hepatic manifestations of MAFLD. We discuss the underlying molecular mechanisms of macrophage activation and polarization as well as cross talk with other cell types such as hepatocytes, hepatic stellate cells and adipose tissue. We conclude with a discussion on the potential translational implications and challenges for macrophage based therapeutics for MAFLD.

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INTRODUCTION

Metabolic associated fatty liver disease (MAFLD), formerly named non-alcoholic fatty liver disease has now reached an “alert” level, an outcome of its high prevalence and its clinical and economic burden. It is estimated that nearly 1 billion people are afflicted globally^[1-3], while projections suggest that the incidence of MAFLD and MAFLD-related complications will continue to burgeon over the next few decades^[4]. By current indications, MAFLD is on trajectory to become the primary cause of hepatocellular carcinoma (HCC) and the primary indication for liver transplantation^[5,6]. Of most concern, around 20 million people who have MAFLD are expected to die of their liver disease. MAFLD not only increases the risk of liver failure and HCC, but also increase the risk for extra-hepatic complications such as type 2 diabetes, cardiovascular disease, chronic kidney disease, osteoporosis, and some type of cancers. The estimated annual medical costs directly attributable to MAFLD is about \$100 billion in the United States and €35 billion in four large European countries (Italy, France, Germany and The United Kingdom)^[7].

The pathogenesis of MAFLD suggests that it is a heterogeneous disease with a variable clinical presentation shaped by interactions between gene and environmental cues^[8,9]. In turn, the clinical presentation is affected by biological and chronological age^[10]. Though MAFLD is classically linked to other metabolic dysfunction and disease such as diabetes and obesity with a shared genetic basis between the conditions^[11], it is now recognised that a significant proportion of patients are non-obese^[1]. The spectrum of disease varies widely ranging from simple steatosis to the presence of inflammation, through to fibrosis, cirrhosis and HCC. Only a proportion (5%-10%) of patients with MAFLD will develop the more severe subtype of steatohepatitis, and some of these will progress to advanced liver fibrosis or cirrhosis, the leading cause of liver related morbidity, and mortality^[12]. While HCC typically develops in the context of cirrhosis, it is increasingly described in patients even in its absence. Hence, the transition from steatosis to steatohepatitis represents a pivotal checkpoint in the natural history of patients with the disease. The current view is that innate immune mechanisms are central to the transition to hepatic inflammation^[13] with macrophages playing a critical role. In this review, we provide a detailed overview on current knowledge on the role of macrophages for liver immune homeostasis and steatohepatitis development and the translational implications and challenges arising from this knowledge.

HEPATIC MACROPHAGES

Hepatic macrophages consist of different cell populations including resident macrophages (aka Kupffer cells) which originate from the yolk sac and function as the dominant liver phagocyte. In addition, bone marrow derived monocytes in the circulation can infiltrate the liver^[14].

The liver has the largest proportion of tissue macrophages among solid organs and it is estimated that for every 100 hepatocytes in a healthy rodent, there are between 20-40 macrophages^[15]. This emphasizes the critical role of liver macrophages in maintaining liver homeostasis^[16], but also indicates the high levels of heterogeneity of this cell population that must exist to enable homeostasis maintenance. Two recent studies employed single cell RNA sequencing to de-convolute hepatic macrophage heterogeneity. In these studies, distinct hepatic macrophage populations with inflammatory and non-inflammatory/immunoregulatory functions were demonstrated^[17]. The second study demonstrated that myeloid cells in liver and bone marrow acquire a functionally distinct inflammatory phenotype in diet induced mouse models of steatohepatitis^[18].

Physiologically, hepatic immune homeostasis is controlled by sinusoidal endothelial cells with liver macrophages part of the liver reticulo-endothelial system^[19]. A recent report suggests that liver macrophages compromise two separate and non-overlapping niches mediating immunosurveillance and that the hepatic

capsule has a cellular network of resident macrophages phenotypically distinct from KCs. These cells sense peritoneal bacteria and restricts hepatic dissemination of these bacteria by promoting neutrophil recruitment to the capsule^[20]. This system creates a dynamic and complex network which therefore constitutes the first line of defence against invading pathogens with contribution of other immune cells such as neutrophils^[16]. At the same time, the upkeep of tissue homeostasis is monitored by the reticuloendothelial system through the production of immunoregulatory cytokines such as interleukin-10 and programmed cell death 1 ligand 1^[21]. Their function includes scavenging bacteria and bacteria-associated products which end up in the liver from the intestine through the portal vein^[22]. However, alterations in this fine-tuned system can lead to different pathological disorders and is strongly implicated in MAFLD development.

MACROPHAGE POLARIZATION

Macrophages polarization is the process whereby macrophages differentiate into sub-phenotypes with specific biological functions in response to signals from their microenvironment including cytokines, growth factors, fatty acids, prostaglandins and pathogen-derived molecules. A simplified classification is the M1 and M2 based activation state^[23,24]. M1 macrophages are pro-inflammatory and antimicrobial and initiate inflammatory processes by expressing high levels of proinflammatory cytokines and producing high amounts of reactive oxygen and nitrogen species^[25]. In contrast, M2 macrophages have anti-inflammatory and reparative functions with high expression of different chemokines compared with M1 macrophages^[24,25]. However, the true complexity of macrophage phenotypes and their regulation is greater than described above and is context dependent and dynamic^[26]. Difficulties in interpreting many published reports results from insufficient characterization of macrophage polarization and function^[14].

MACROPHAGES AND THEIR ROLES IN MAFLD

Macrophages, steatosis development, and the transition to steatohepatitis

Macrophages are highly versatile, while extensive experimental and clinical data indicates that they play a central role in MAFLD development with pro-inflammatory macrophages determining disease severity^[14]. In human MAFLD, portal macrophage infiltration is observed at an early stage before inflammation is evident and is associated with progressive disease^[27]. Another study of young adult Koreans showed an increase in the numbers of CD68⁺ Kupffer cells in biopsy samples from patients with steatohepatitis compared to those with steatosis^[28]. Similarly, an increase of activated macrophages was found in children with MAFLD and these were located in the vicinity of damaged hepatocytes^[29].

Additional evidence for a central role for macrophages in steatohepatitis comes from experimental models. In dietary mouse models, an increase in hepatic macrophages that produce pro-inflammatory cytokines was observed in high-fat diet (HFD) and methionine-choline-deficient (MCD) diet fed mice^[30,31]. Moreover, the production of these inflammatory mediators was increased after 4 wk in mice fed a MCD diet and decreased subsequently, suggesting a determinant role in the transition to steatohepatitis^[32]. In addition, immune responses are innately different between mouse strains; T helper 1 cell and M1 responses are observed in C57BL/6 mice whereas T helper 2 cell and M2 response are observed in BALB/c mice^[33]. Feeding these two strains an MCD diet showed that steatosis and hepatic inflammation was more induced in M1-prone C57BL/6 mice compared with the M2 prone BALB/c strain^[34]. Furthermore, the use of clodronate liposomes or gadolinium chloride to deplete macrophages protected mice from steatosis development^[35].

Pro-inflammatory macrophages have also been found to induce hepatic insulin resistance and decrease hepatocyte responsiveness to insulin by attenuating phosphorylation of the insulin receptor substrate 1 in hepatocytes^[36]. Thus, silencing of *NF-κB* specifically in Kupffer cells improves insulin sensitivity and decreases cytokine secretion in a HFD-fed model further suggesting a crucial role for them in hepatic insulin resistance^[37]. Another report found that HFD-fed mice injected with clodronate to deplete Kupffer cells had decreased steatosis and steatohepatitis *via* reducing interleukin-1β-dependent suppression of peroxisome proliferator-activated receptor-α (PPARα)^[38]. The latter is suggested to have an anti-inflammatory role in liver and adipose tissue^[39]. Along the same line, depletion of Kupffer cells leads to reduction of inflammatory cytokine expression and decreases inflammation and liver

cell death^[40]. Another suggested mechanism is of p38 mitogen-activated protein kinases being upregulated in the liver of patients with MAFLD in multiple diet induced steatohepatitis mouse models. Macrophage p38 induces M1 polarization and pro-inflammatory cytokine secretion promoting the progression to steatohepatitis^[41].

Macrophages and liver fibrosis

In virtually all chronic liver diseases, there is no fibrosis without preceding or concomitant inflammation. Liver macrophages play a pivotal role in fibrosis progression with these cells and hepatic stellate cells (HSCs, the major producer of extracellular matrix) exhibiting bidirectional signalling^[42]. Thus, chemokines/cytokines from HSCs augment macrophage infiltration, while macrophages amplify inflammation, contribute to maintain the fibrogenic phenotype and promotes HSC survival. Macrophages also play a dominant role in fibrosis resolution. Alternatively activated M2 macrophages correlate with hepatic injury in MAFLD^[31] orchestrating a fibrosis response favouring liver remodelling and tissue repair by producing transforming growth factor- β and platelet-derived growth factor among other proteins^[43].

Distinct monocytes/macrophage populations can be found in human and mouse liver based on levels of Ly-6C (Gr1) or CD14/CD16 expression, in murine and humans respectively. In humans it includes “classical” CD14⁺⁺CD16⁻ and “non-classical” CD14⁺CD16⁺ monocytes/macrophages as well as CD16⁺⁺ cells. Notably, fibrosis is associated with preferential enrichment of CD14⁺CD16⁺ cells or its functional counterpart Ly-6C^{hi} in mouse^[44,45]. These cells activate HSCs *in vitro*, partially dependent on transforming growth factor (TGF)- β release^[44,45]. Conversely, the production of soluble mediators such as CC-chemokine ligand 2 (also known as MCP1) and macrophage colony-stimulating factor by activated HSCs augments inflammatory cell infiltration to initiate and maintain myofibroblast activation^[46]. Ly-6C^{lo} cells have been identified as a feature of “restorative” hepatic macrophages in mice^[47] but the exact counterpart of these in humans remains to be clarified. In mouse models, autophagy gene *Atg5* in the myeloid lineage *Atg5*(fl/fl) *LysM*-Cre knockout mice have demonstrated that macrophage autophagy attenuates liver fibrosis^[48]. Additionally, immune cell subset differentiation can perpetuate or restrict hepatic injury^[19].

In sum, at different stages of hepatic injury, both resident Kupffer cells and freshly recruited monocyte-derived macrophages play critical roles in the regulation of inflammation, fibrosis and fibrolysis^[49].

Macrophages and HCC

MAFLD can increase the risk of HCC even in the absence of cirrhosis^[50]. Tumor associated macrophages secrete inflammatory cytokines such as tumour necrosis factor- α and growth factors such as vascular endothelial growth factor and TGF- β that are involved in angiogenesis and contribute to tumor development, progression, and metastasis^[51]. Toll like receptor (TLR) 4 but not TLR2 on macrophages has also been demonstrated to contribute to steatohepatitis-related HCC in mice by inducing proinflammatory cytokines and the proliferation of HCC and cancer progenitor cells^[52]. Another mechanistic study suggests that obesity-associated oxidative stress increases STAT-1 and STAT-3 signaling which can independently contribute to the pathogenesis of steatohepatitis, fibrosis, and HCC in mouse models^[53].

MACROPHAGES AND MAFLD EXTRA-HEPATIC MANIFESTATIONS

The consequences and complications of MAFLD are not limited to liver, but also extend to include various extra-hepatic organ involvement including type 2 diabetes mellitus, chronic kidney disease, osteoporosis, hypothyroidism, some type of cancers, and cardiovascular disease^[54]. The mechanisms that contribute to this heightened risk for cardiovascular disease and type 2 diabetes mellitus risk are poorly understood.

Disordered myelopoiesis and macrophage-mediated inflammation was recently suggested as a plausible overarching mechanism linking MAFLD to cardiovascular diseases^[55]. Soluble CD163, a macrophage activation marker correlates with liver injury^[56] with similar finding reported for CVD risk^[57]. As discussed above, apart from their central role in progression to steatohepatitis and fibrosis, macrophages are known to enter to plaques and promote lesion progression, instability and rupture^[58].

FACTORS REGULATING MACROPHAGE PHENOTYPE AND POLARIZATION IN MAFLD

Dietary factors

Nutrition and the intracellular metabolism of macrophages are a key regulator of their function and can determine the skew of macrophages towards a pro or anti-inflammatory phenotype^[59]. For example, dietary cholesterol differentially shapes the transcriptome of Kupffer cells and infiltrating macrophages during steatohepatitis progression towards a pro-inflammatory phenotype^[60]. Similarly, a HFD induces macrophage polarization and aggravates the liver inflammatory microenvironment and cancer progression in a zebrafish model of MAFLD-associated HCC; this effect was reversed by metformin^[61]. Conversely, exercise training and weight loss suppress macrophage activation as assessed by soluble CD163 (sCD163)^[62,63]. Similarly, a reduction in sCD163 was noticed following bariatric surgery which was accompanied by improvements in insulin sensitivity and liver enzymes^[64]. Functionally, endolysosomal lipid trapping and accumulation in Kupffer cells in response to high fat diet feeding upregulates hepatic inflammatory gene expression^[65,66] while fatty acids can increase mitochondrial DNA release causing activation of NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes in Kupffer cells^[67]. In contrast, vitamin D receptor activation in hepatic macrophages improves insulin resistance, steatosis and hepatic inflammation through the induction of an anti-inflammatory phenotype^[68].

Hematopoiesis in bone marrow is influenced by environmental cues including diet. Myelopoiesis is tightly regulated in bone marrow^[69], governing the generation of mature cells *via* sequential progression from HSCs to differentiated cells including monocytes and macrophages that share a distinct committed progenitor^[70,71]. Dysregulation of hematopoiesis switches the protective response to pro-inflammatory myelopoiesis in the marrow and governs ongoing and future inflammatory responses^[72-74]. A recent study showed that bone marrow derived macrophages from western diet fed mice have an inflammatory activation profile compared to those from normal chow fed mice^[48].

Thus, dietary factors play a role in determining macrophage polarisation and activation as well as in shaping myelopoiesis. Macrophage polarisation and activation is shown in [Figure 1](#).

The Gut–liver axis

Dysbiosis of the gut microbiota is a factor contributing to the development and progression of fatty liver disease^[75]. Dysbiosis affects hepatic macrophage activation and polarization through multiple potential mechanisms. For example, the gut microbiome plays a central regulatory role in host metabolism whereby alterations in the metabolic outputs of intestinal microbiota affect macrophage polarization^[75]. A recent study using metabolomics analysis of cecal and fecal material from germ-free and conventionally raised HFD fed mice identified a gut microbiota-derived metabolite indole-3-acetate that directly modulates inflammatory responses in macrophages and dampens the release of pro-inflammatory cytokines^[76]. Mechanistically, these effects were mediated through the aryl-hydrocarbon receptor, a transcription factor that regulates responses to multiple environmental signals including dietary factors^[76]. In the same vein, ARNT mRNA expression is downregulated in liver tissues from MAFLD patients, while deletion of ARNT in mouse myeloid cells leads to steatohepatitis^[77]. Similarly, the dysregulation of gut microbiota of infants of obese mothers leads to impaired macrophage function including cytokine production, phagocytosis and adaptation to various stimuli that ultimately increases susceptibility to MAFLD^[78,79].

Another mechanism exists whereby alterations in the intestinal permeability in patients and murine models of MAFLD that is mediated by a western diet, leads to increases in the circulating levels of bacterial products including lipopolysaccharide (LPS). This leads to activation of TLRs, a sensor for these products^[80] and results in macrophage activation and the initiation of injury^[81-85]. Serum LPS and hepatic TLR4⁺ macrophages are higher in steatohepatitis patients compared to those with MAFLD or control subjects^[86]. Similarly hepatic TLR 2, TLR4 and TLR9 expression was upregulated in human and murine steatohepatitis but not in simple steatosis; expression was localized to inflammatory cells, particularly macrophages^[87]. Finally, Tlr4 and Tlr9 KO mice are protected from steatohepatitis induced by different diet models of MAFLD such as MCD feeding or a cholesterol rich diet^[88,89]. Notably, the TLR effects also involves interaction and activation with other intersecting pathways with a role in inflammation. In a recent study, LPS treatment induced the accumulation of yes-associated protein (YAP) in Kupffer cells, a transcription

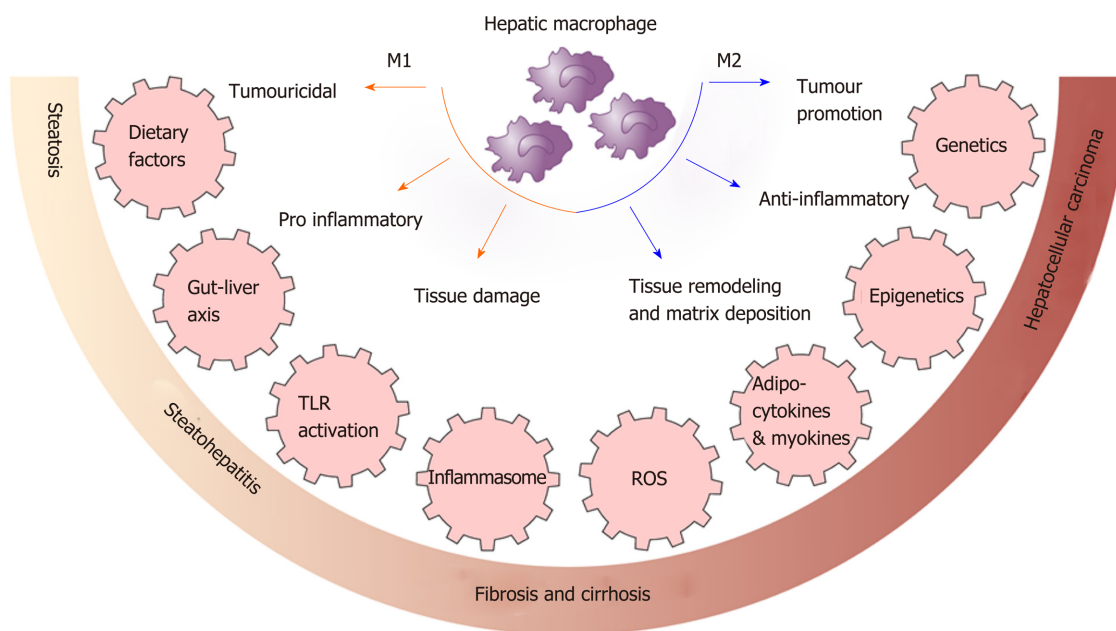


Figure 1 Macrophage polarisation and activation. Macrophages polarize to sub-phenotypes with specific biological functions in response to signals from their microenvironment. These include signals from adipocytokines and myokines, the gut liver axis and toll-like receptor activation, damaged hepatocytes, reactive oxygen species, genetic and epigenetic factors. The process of macrophage activation is a critical determinant of disease progression and is a target for potential treatment. ROS: Reactive oxygen species; TLR: Toll-like receptor.

coactivator that plays a role in the Hippo-YAP pathway and is implicated in the immune response^[90]. YAP accumulation in Kupffer cells in steatohepatitis enhances the production of pro-inflammatory cytokines^[90]. Furthermore, the elevated LPS in MAFLD^[84,85] provides a critical hit for sustained inflammasome activation in macrophages^[91].

TLR4 stimulation also induces alterations in lipid homeostasis^[92] and macrophage activation as assessed by sCD163 as was found in a small trial that included 8 healthy male subjects^[93]. In that study, sCD163 correlated with accelerated lipolysis following LPS exposure and enhanced mitochondrial reactive oxygen species (ROS) generation, a trigger for inflammasome activation^[94].

Collectively, gut dysbiosis is implicated in macrophage activation and hepatic inflammation through multiple mechanisms including by alterations in the metabolic outputs of microbiota, increased bacterial products generation and the activation of TLRs and inflammasomes. The gut liver axis in metabolic associated fatty liver disease is shown in **Figure 2**.

Adipocytokines and myokines

Both adipocytokines and myokines play an important regulatory role in macrophage activation and polarisation. The anti-inflammatory effects of adipocyte-derived adiponectin can partially be attributed to promoting the polarization of macrophages toward an anti-inflammatory phenotype^[95] as well as promoting macrophage tolerance to pro-inflammatory stimuli^[96]. Adiponectin KO mice are more prone to steatohepatitis, while adiponectin administration attenuates steatohepatitis progression by reducing macrophage infiltration and skewing polarization towards an anti-inflammatory phenotype^[97]. Levels of leptin are increased in MAFLD and this regulates macrophage phenotype including Kupffer cell activation, inflammatory phenotype and sensitivity to LPS-induced inflammatory cytokine secretion and acquisition of a fibrogenic phenotype^[98-100]. A previous study showed that Kupffer cells likely mediate leptin-induced liver fibrosis effects by inducing the upregulation of fibrogenic gene expression such as that of TGFβ1 and connective tissue growth factor^[100].

Levels of fibronectin type III domain-containing 5 (FNDC5) a myokine with favourable metabolic effects is decreased in MAFLD and correlates with the severity of hepatic steatosis^[101]. FNDC5 attenuates adipose tissue insulin resistance and inflammation *via* AMP-activated protein kinase-induced macrophage polarization in a high fat diet mice model^[102]. Another member of the fibronectin type III domain family of proteins is FNDC4 a secreted factor with a high homology to FNDC5. This was demonstrated to have anti-inflammatory effects on macrophages by reducing

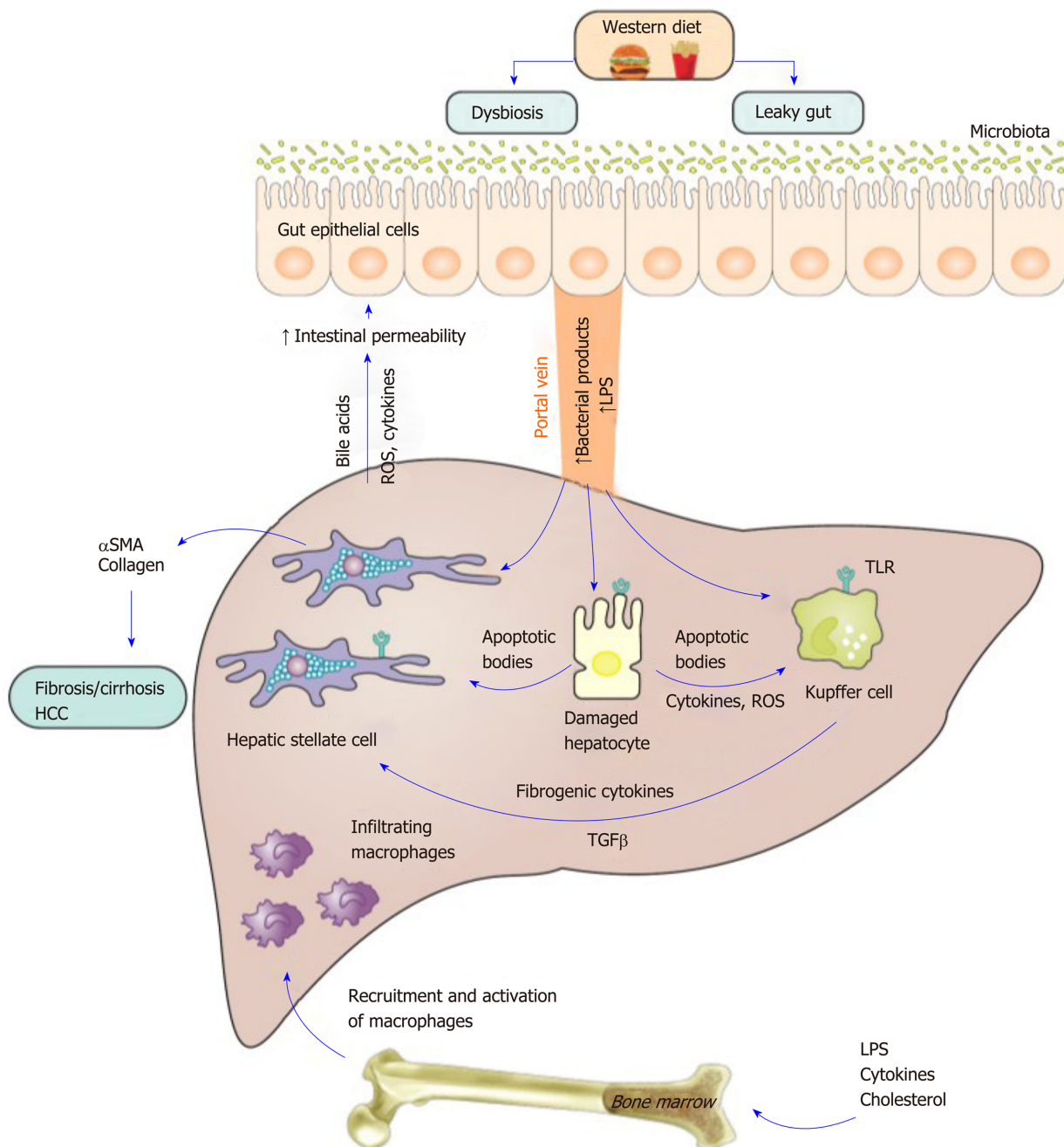


Figure 2 The gut liver axis in metabolic associated fatty liver disease and the central role of macrophages. A western diet alters gut permeability and causes dysbiosis. This increases hepatic exposure to lipopolysaccharide and bacterial products and leads to toll-like receptor activation in hepatocytes, hepatic stellate cells and macrophages; the latter are the main responsive cells. Hepatocyte injury leads to the generation of apoptotic bodies, reactive oxygen species and cytokines. Engulfment of apoptotic bodies by macrophages increases expression of death ligands such as tumor-necrosis-factor-related apoptosis-inducing ligand, Fas, and tumour necrosis factor- α leading to a feed-forward loop that promotes further hepatocyte apoptosis. Engulfment of apoptotic bodies by hepatic stellate cells promotes their activation and the secretion of transforming growth factor β 1 and extracellular matrix, promoting fibrosis and subsequently cirrhosis. Activated macrophages also lead to increased activation of stellate cells. Cholesterol, bacterial products and cytokines can stimulate myelopoiesis in bone marrow. Infiltrating monocytes lead to amplification of the inflammatory response. Increased bile acid and reactive oxygen species production by the injured liver also contributes to damage to gut epithelial cells and to detrimental alterations in microbiota setting up a vicious cycle of injury. LPS: Lipopolysaccharide; TLR: Toll-like receptor; TGF- β : Transforming growth factor β ; HCC: Hepatocellular carcinoma.

proinflammatory chemokine expression and dampening macrophage activity^[103]. The role of FNDC4 in MAFLD is unknown. In sum, adipocytokines and myokines represent another crucial regulator of macrophage biology in the context of metabolic disorders.

Damaged hepatocytes and ROS and macrophage activation

Damaged hepatocytes are a trigger for macrophage activation. Data from mice fed a HFD suggest that steatotic hepatocytes promote the release of pro-inflammatory cytokines by macrophages, indicating that hepatocyte damage elicits macrophage

activation^[104]. In turn, apoptotic body engulfment by Kupffer cells is a potent trigger for inflammation and fibrosis and for activation of macrophages *via* pattern recognition receptors such as TLRs^[105]. Trying to elucidate the link between damaged hepatocytes and macrophage activation and inflammation, a recent study has suggested that proapoptotic lipotoxic signaling by death receptor 5 (also known as tumor necrosis factor receptor superfamily member 10b) induces the release of extracellular vesicles from hepatocytes. These in turn activate macrophages and promotes an inflammatory phenotype^[106]. Similarly, another study showed that hepatocytes release ceramide-enriched pro-inflammatory extracellular vesicles under lipotoxic conditions that promote macrophage recruitment to the liver^[107]. Recent reports have also demonstrated increased expression of the G protein-coupled transmembrane receptor Smoothened and glioma-associated oncogene (Gli) transcription factors (components of the hedgehog signaling pathway) in primary hepatocytes from damaged livers^[108]. Pharmacological and genetic liver specific inhibition of Smoothened prevented hepatic inflammation in MAFLD models through decreased macrophage recruitment and activation^[109].

ROS are another potential mechanism for macrophage activation. ROS impacts Kupffer cells both directly and indirectly. ROS prompts tumour necrosis factor- α generation in Kupffer cells and they can increase the susceptibility of these cells to endotoxin^[110,111].

Genetic variants that influence macrophage function and thereby liver inflammation

There is strong evidence that MAFLD has high heritability with a shared genetic basis between MAFLD and other liver diseases as well as with other metabolic disorders^[11]. The genetic basis of macrophage activation is still not known, however several genetic variants implicated in MAFLD have been demonstrated to regulate macrophage phenotype. For example, the interferon lambda 3/4 (*IFNL3/IFNL4*) genotype is strongly associated with hepatic inflammation and fibrosis in MAFLD as well as hepatitis C and B^[112,113] and scores incorporating these variants with other clinical variables can predict liver fibrosis in patients^[114]. A correlation between *IFNL3/IFNL4* genotype and hepatic macrophage infiltration as well as macrophage activation as assessed by the activation marker sCD163 has been reported^[115-119]. Recently, increases in hepatic *IFNL3* expression in liver tissues from patients with MAFLD compared to controls was also reported^[120]. *IFNL3* skews macrophages toward a proinflammatory phenotype orchestrating their interaction with other immune cells including T-cells to mediate hepatic inflammation^[120].

Another example is of variants in the Membrane Bound O-Acyltransferase Domain Containing 7 (*MBOAT7*) gene that encodes an acyltransferase enzyme and catalyses the transfer of an acyl-CoA to lysophosphatidylinositol. This regulates the availability of arachidonic acid for pro-inflammatory eicosanoids production^[121-124]. A variant (rs641738) in the *MBOAT7* gene is associated with liver injury in MAFLD as well as in viral hepatitis^[121-124]. *MBOAT7* is robustly expressed by inflammatory cells including macrophages and this variant associates with macrophage activation as assessed by sCD163; the detailed mechanisms for this association are unknown^[121-124]. A recent study showed that *Mboat7* loss of function promotes the progression of MAFLD and hepatic inflammatory and fibrotic phenotypes in response to a high fat diet^[125].

Another example is a variant (rs4374383) in the MER Proto-Oncogene, Tyrosine Kinase (*MERTK*) gene, a member of the Tyro-Axl-MerTK family of receptor tyrosine kinases. By a genome-wide association study this polymorphism has been associated with fibrosis in patients with hepatitis C^[126]. Similar findings were later observed in patients with MAFLD^[127]. A recent study has shown that this variant regulates *MERTK* expression in circulating and hepatic macrophages and that total and myeloid specific *MERTK* deficiency decreased liver fibrosis in mice^[128].

Epigenetic regulation

Epigenetic modifications include DNA methylation, histone modification and non-coding RNA^[8]. Growing evidence suggests an important role for epigenetic mechanisms in the regulation of macrophage phenotype, polarization, inflammatory and profibrotic gene production. A recent work used reduced representation bisulfite sequencing in a mouse carbon tetrachloride (CCl₄) fibrosis model and identified that hypermethylation of proline-serine-threonine-phosphatase-interacting protein2 links it to hepatic fibrosis^[129]. In another study, the histone methyltransferase Suv39h2 was found to contribute to steatohepatitis in mouse models through suppression of PPAR γ . This leads to macrophage polarisation towards a proinflammatory M1 over an anti-inflammatory M2 phenotype^[130]. miRNAs are also implicated in macrophage activation and polarisation. For example, lipotoxic hepatocyte-derived exosomal miR-192-5p plays a pivotal role in the activation of proinflammatory macrophages and in disease progression of MAFLD *via* Rictor/Akt/FoxO1 signaling^[131].

ADIPOSE TISSUE AND FATTY LIVER CROSS TALK: ROLE OF MACROPHAGES

A pivotal role for macrophages in the crosstalk between adipose tissue and liver in fatty liver disease has been suggested. Thus, adipose tissue insulin resistance and free fatty acid flux to liver activates hepatic macrophages in MAFLD independent of obesity and diabetes^[132]. It has also been suggested that adipose tissue inflammation might precede hepatic inflammation since in high fat and cholesterol diet fed mice, the upregulation of genes associated with macrophage recruitment and inflammation occurred earlier in adipose tissue compared to the liver^[133]. During obesity, both adipose tissue macrophage (ATM) numbers and activation are enhanced^[134]. In humans, in a cohort of obese patients undergoing bariatric surgery, ATMs from patients with MAFLD and steatohepatitis produced higher levels of inflammatory cytokines compared to controls^[135]. In animal models, ablation of these ATMs led to normalization of whole-body insulin sensitivity^[136] while ATMs from obese visceral adipose tissue amplified hepatic inflammation through augmentation of hepatic macrophage infiltration^[137].

TRANSLATIONAL IMPLICATIONS

Although MAFLD is a major clinical problem, to date there are no approved treatments. Recently, obeticholic acid was reported to improve MAFLD related fibrosis and will likely be the first drug to be approved for its treatment^[138,139]. With multiple other investigational drugs in Phase 3 clinical trials, it can be expected that other drugs will follow and be approved. However, the efficacy of these drugs appears modest suggesting that more novel approaches to drug development are required^[140]. Given the central role of macrophages in the transition to steatohepatitis and to fibrosis, macrophages might be a suitable target for therapy and as a biomarker of disease severity, as discussed below. The central role of macrophages is shown in [Figure 2](#).

Macrophage based diagnostic biomarkers

Soluble CD163, a macrophage activation marker was reported to correlate with liver injury and demonstrated good predictive ability for advanced fibrosis (\geq F3) with an area under the receiver operating curve (AUROC) of 0.77 and 0.80 in two independent cohorts. This was further increased in combination with the MAFLD fibrosis score (AUROC of 0.83 for both cohorts)^[56]. However, macrophage activation markers such as sCD163 and soluble mannose receptor demonstrated poor associations with liver histology in two cross-sectional paediatric MAFLD cohorts ($n=155$ and 36) suggesting a differential role for macrophage activation in adult and paediatric disease or perhaps indicates that disease severity is different between adult and paediatric patients at least in the cohorts examined^[141].

A recent study also suggested that the circulating activity of adenosine deaminase (a macrophage-derived deaminase that converts adenosine or deoxyadenosine to inosine and derivatives) can predict MAFLD cirrhosis, advanced fibrosis (\geq F3), and significant fibrosis (\geq F2) with AUROCs of 0.94, 0.82, and 0.84, respectively^[142]. However, the molecular mechanisms for this association are yet to be determined.

Macrophage based therapeutic targets

Some macrophage-targeted therapies for liver diseases have been investigated in the clinic^[143]. One of these is limiting monocyte and macrophage recruitment. Many of the pathways characterized in mice for monocyte recruitment are also strongly regulated in patients with liver diseases suggesting well conserved mechanisms across species. For instance, cenicriviroc, an oral dual inhibitor of the chemokine receptor C-C ligand/ receptor 2 and 5 pathway (inhibits hepatic monocyte infiltration)^[144] was evaluated in a phase 2b clinical trial in patients with MAFLD and fibrosis. Early results suggest that patients receiving cenicriviroc oral therapy were twice as likely to have improvement of fibrosis (without worsening of steatohepatitis) after 1 year of follow up^[141]. However, the differences did not appear to be significant by the second year of follow up.

Another approach for treatment is to promote anti-inflammatory macrophage polarization. The PPAR family that includes α , β/δ , and γ is a member of the nuclear receptor superfamily and polarizes macrophages towards an anti-inflammatory state. This can explain at least partially their role in MAFLD^[145,146]. For example, among modulators of the PPARs, PPAR γ agonists such as pioglitazone have insulin sensitizing effects but also induces polarization of macrophages towards an anti-

inflammatory phenotype thereby improving hepatic steatosis^[145,146]. Similarly, the PPAR α /PPAR γ agonist saroglitazar reverses fibrosis in MAFLD^[147] and elafibranor, an agonist of PPAR α and δ attenuates hepatic inflammation without worsening fibrosis^[148]. PPAR δ induces polarization of macrophages towards an anti-inflammatory M2 phenotype^[145] while elafibranor decreases hepatic macrophage infiltration in animal models of MAFLD^[149].

An alternative approach is targeting activating signals for macrophages. For example, NLRP3 inflammasome pharmacological blockade using MCC950 attenuates hepatic inflammation and fibrosis in mouse models and reduces the numbers of macrophages in liver^[150]. Currently, a clinical trial (NCT03676231) using an inflammasome inhibitor SGM-1019 is recruiting. TLR4 is a key regulator of MAFLD^[151] however the results of a phase-2 study failed to discern a beneficial effect of JKB-121, a small molecule TLR-4 receptor antagonist in human MAFLD^[152].

The growing body of knowledge on immunometabolism and epigenetic regulation indicates that metabolic reprogramming and epigenetic regulation may be a target for regulating macrophage responses such as polarization and activation. Two recent reports demonstrate that digoxin improves steatohepatitis in mice models through regulation of the pyruvate kinase muscle isozyme M2-hypoxia-inducible factors axis in hepatocytes and macrophages^[153,154]. Pyruvate kinase muscle isozyme M2 is a rate limiting glycolytic enzyme that catalyzes the final step in glycolysis^[155] and promotes NLRP3 and AIM2 inflammasome activation in macrophages^[156].

In a recent study of mice fed a MCD diet, miR-141 and miR-200c deficiency attenuated hepatic steatosis and inflammation *via* multiple mechanisms including reprogramming of macrophages toward anti-inflammatory phenotype^[157]. Although several epigenetic drugs are currently approved for treating different type of cancers, data are still limited on effects of these drugs on macrophage activation and polarisation. Histone deacetylase inhibition was demonstrated to improve post-myocardial infarction healing through modulating macrophage polarization^[158]. Another study showed that targeting histone deacetylases in myeloid cells using CHR-4487 (ESM-HDAC528) a small molecule that can inhibits their inflammatory phenotype has a limited impact in atherosclerosis^[159]; there is no data on MAFLD.

CHALLENGES AND OPEN QUESTIONS

Although targeting macrophages is attractive, a major obstacle for the development of therapies is that of macrophage heterogeneity and differences between mice and humans^[143,160]. Macrophages exhibit remarkable plasticity and phenotypic heterogeneity and different subsets have distinct and sometimes opposite properties (pro- *vs* anti-inflammatory, pro- *vs* antifibrotic). Further, environmental factors including cytokines control the polarization of macrophages but are not well defined. It is very likely that the functionality of hepatic macrophage subsets is also influenced by the nature of the underlying liver disease and this will hinder their use in an etiology independent manner. Another challenge for translating findings from murine models to humans is the fact that murine and human monocyte/macrophage subpopulations do not share the same surface-marker profiles. However new technologies including single cell RNA-Seq can help to de-convolute macrophage heterogeneity and may enable the development of specific therapies. Further, clarifying the basis of modulation of epigenetic and metabolic programs in macrophages can guide therapeutics. Delivery modalities represents another challenge, with multiple delivery platforms having been explored including nanoparticles, liposomes and oligopeptide complexes. These methods can be used to target macrophages for delivery of agents such as gene-silencing siRNA and miRNA inhibitors. While CRISPR-Cas9 technology might revolutionize medicine, their application for modulating macrophage biology is not well explored.

CONCLUSION

Macrophages play a central role at all stages of MAFLD and contributes to the pathology in extrahepatic sites that are simultaneously affected. Thus, macrophage directed therapeutics have unique potential in MAFLD, but their heterogeneity represents a challenge. Metabolic and epigenetic programming and gene-based therapies are attractive approaches to manipulate macrophage function for clinical benefit, though the optimal delivery methods are still not defined. In the future, combined approaches consisting of multiple drugs that target different key pathways are likely to provide a better strategy to treat MAFLD. It remains to be elucidated

which combinations should to be used, but macrophage targeted therapies are one viable option.

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Regulation of macrophage activation in the liver after acute injury: Role of the fibrinolytic system

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Abstract

The liver functions, in part, to prevent exposure of the body to potentially harmful substances ingested in the diet. While it is highly efficient at accomplishing this, it is frequently prone to liver injury due to the biotransformation of xenobiotics into toxic metabolites. To counter this injury, the liver has evolved a unique capacity to rapidly and efficiently repair itself. Successful resolution of acute liver injury relies on hepatic macrophage populations that orchestrate the reparative response. After injury, Kupffer cells, the resident macrophages of the liver, become activated and secrete proinflammatory cytokines. These cytokines recruit other immune cells, including monocyte-derived macrophages, to the liver where they contribute to the repair process. Monocyte-derived macrophages traffic into the necrotic foci where they rapidly phagocytose dead cell debris. Simultaneous with this process, these cells change phenotype from a proinflammatory macrophage to a pro-restorative macrophage that produce pro-mitogenic growth factors and anti-inflammatory cytokines. Ultimately this process triggers resolution of inflammation, and along with proliferation of other hepatic cells, restores the liver architecture and function. While the mechanisms regulating specific macrophage functions during repair remain to be elucidated, recent studies indicate a key role for the fibrinolytic system in coordinating macrophage function during repair. In this review, we will highlight the function and role of hepatic macrophages in repair after acute liver injury, and will discuss the role of the fibrinolytic enzyme, plasmin, in regulation of these various processes.

Key words: Macrophage; Plasmin; Acetaminophen; Liver injury; Liver repair

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Core tip: Macrophages contribute to repair of the liver after injury. After injury, Kupffer cells release cytokines that recruit monocyte-derived macrophages that phagocytose dead cell debris. These cells switch phenotype becoming pro-restorative macrophages that

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terminate cytokine synthesis and produce pro-mitogenic growth factors that facilitate liver repair.

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INTRODUCTION

By virtue of its location in the circulatory system, the liver acts as an essential barrier to prevent the systemic dissemination of potentially deadly pathogens and toxic xenobiotics that enter the portal circulation from the gastrointestinal tract. To carry out this function, the liver is home to an extensive network of resident and patrolling immune cells that target and kill pathogens. Further, hepatocytes, the primary functional cell type of the liver, express a battery of xenobiotic metabolizing enzymes that detoxify potentially harmful chemicals and target them for excretion. While the liver is highly efficient at preventing systemic exposure to toxic substances, it is prone to injury from reactive metabolites generated from the biotransformation of xenobiotics. To counter this, the liver has evolved a remarkable capacity for repair even after extensive injury. In cases of chronic injury, however, multiple cycles of injury and repair can ultimately lead to scar formation, also called fibrosis or in severe cases, cirrhosis. A cell type that is intimately involved in all aspects of liver injury and repair is the macrophage. This specialized cell of the immune system modifies its phenotype in response to local cues generated in the hepatic microenvironment. Through this action, macrophages can take on a number of diverse functions after liver injury, including killing and phagocytosing bacteria, producing cytokines that regulate recruitment and function of other immune cells, and/or producing tissue reparative growth factors. In the following review, we will discuss the role of macrophages in triggering the response to liver injury and highlight their role in various aspects of liver repair, particularly after acetaminophen (APAP)-induced liver injury. In addition, we will discuss the critical role of the fibrinolytic system in regulation of macrophage function after acute liver injury and during repair.

HEPATIC MACROPHAGES

Kupffer cells (KC), which are resident to the liver, are cells of the myeloid lineage normally present on the luminal side of the hepatic sinusoid. These cells detect, phagocytose, and degrade foreign materials, pathogens, and cellular debris that enter the liver through the portal circulation (for a comprehensive review of KC function^[1]). KC arise from progenitor stem cells generated in the fetal yolk-sac early during development^[2]. These cells migrate to the liver where they become fully functioning KC. During homeostasis or after toxin-induced liver injury, these cells are replenished through the local proliferation of mature KC^[3]. Although the stimulus for local proliferation of KC is not fully known, studies suggest that colony stimulating factors may contribute to this process^[4]. Under conditions where a substantial loss of KC occurs, such as after exposure to lethal irradiation, these cells can be replenished from bone marrow progenitors^[5,6]. For instance, our recent studies determined that after lethal irradiation and bone marrow transplantation, approximately 40% of KC are replaced by macrophages originating from bone marrow which is consistent with findings by others^[5,7]. During this process, monocytes are recruited from the circulation and take up residence within the hepatic sinusoids^[8]. Overtime, local cues generated in the hepatic microenvironment reprograms these cells to become KC that are nearly indistinguishable from their predecessors. Recent studies suggest that this process requires Notch and transforming growth factor- β signals generated by sinusoidal endothelial cells and requires agonists of the liver X receptor^[8].

In addition to KC, a second, distinct population of hepatic macrophages characterized by selective expression of Cx3cr1 was recently identified that resides proximal to the Glisson's capsule^[9]. Studies suggest that these macrophages provide a barrier against invasion of pathogens from the peritoneal cavity into the liver^[9].

Remarkably, these macrophages appear to extend protrusions into the peritoneal cavity where they can sense and respond to bacteria^[9]. Unlike KC, this macrophage population is replenished from circulating monocytes generated from myeloid progenitors in the bone marrow^[3,9]. A similar population of Cx3cr1⁺ macrophages is also located proximal to blood vessels in the liver^[3]. These macrophages may function as a last line of defense against dissemination of bacteria into the systemic vasculature. The mechanisms controlling recruitment and specialization of these macrophages remains to be investigated.

MACROPHAGE FUNCTION AFTER ACUTE LIVER INJURY

Much of what we know regarding macrophage function in liver repair derives from studies investigating APAP-induced liver injury. APAP is a commonly used analgesic and antipyretic. Although APAP is considered safe at low, therapeutic doses (*i.e.*, 4 g/d), APAP overdose, either accidental or intentional, results in approximately 56000 emergency room visits, 26000 hospitalizations and 458 deaths each year making it responsible for nearly 50% of all cases of acute liver failure (ALF) in the United States^[10,11]. At low doses, APAP is rapidly metabolized by glucuronidation or sulfation in the liver and excreted into the urine by the kidneys. APAP can also be oxidized by cytochrome P450s to the hepatotoxic intermediate, N-acetyl-*p*-benzoquinone imine (NAPQI), however, at therapeutic doses, NAPQI is rapidly detoxified by glutathione. At toxic doses of APAP, glucuronidation and sulfation pathways become saturated, which shifts metabolism towards oxidation to NAPQI. High concentrations of NAPQI ultimately deplete cellular glutathione leading to the accumulation of NAPQI, which forms protein adducts stimulating oxidative stress, mitochondrial permeability transition, loss of ATP, and ultimately hepatocyte necrosis^[12,13]. Within hours of hepatocyte injury, however, a robust reparative response is initiated^[14]. During this process, hepatic macrophages become activated and release proinflammatory cytokines that stimulate the recruitment of various immune cell types^[4]. Pro-mitogenic cytokines and growth factors are also released stimulating sinusoidal endothelial cell and hepatocyte proliferation. As cell proliferation proceeds, dead cell debris is removed from the liver, and anti-inflammatory cytokines are produced causing resolution of inflammation. Remarkably, within days of the initial insult, liver structure and function are fully restored.

CONTRIBUTION OF KC TO APAP-INDUCED LIVER INJURY AND REPAIR

Macrophages perform several key functions in the liver after APAP overdose, including production of immunomodulatory cytokines, phagocytosis of dead cell debris, and production of pro-mitogenic growth factors^[15]. While it is well established that macrophages perform these critical functions, the importance of KC to these processes remains a matter of debate. Early studies indicated a pathogenic role for KC after APAP overdose. In these studies, treatment of mice with the macrophage inhibitor, gadolinium chloride, protected against APAP hepatotoxicity^[16]. Subsequent studies indicated that inhibition of KC with gadolinium chloride prevented production of reactive oxygen species and peroxynitrite after APAP overdose, leading to reduced liver toxicity^[17]. Accordingly, it was concluded that KC were critical for liver toxicity after APAP overdose^[17]. More recent studies, however, which used clodronate-containing liposomes to fully deplete KC, demonstrated that KC depletion exacerbated hepatic necrosis at 8 and 24 h after an acutely toxic dose of APAP^[18]. Further investigation revealed that KC depletion was associated with a reduction in the anti-inflammatory cytokine, IL-10^[18]. Consistent with this finding, subsequent studies revealed that IL-10 knockout mice had increased liver toxicity and mortality after APAP overdose^[19]. Based upon these findings, it was concluded that IL-10, released from KC, protected the liver from toxicity after APAP overdose. Although these studies demonstrate that KC are an important source of anti-inflammatory cytokines (*e.g.*, IL-10), studies have also shown that KC are an important source of proinflammatory mediators after APAP overdose. In support of this, studies using a murine model of APAP-induced liver injury, demonstrated that KC release several proinflammatory cytokines, including IL-1 β , tumor necrosis factor (TNF)- α , and Ccl2, by 6 h after APAP challenge^[20,21]. Although KC appear important for early cytokine induction after APAP overdose, by 24 h after APAP treatment, the population of resident KC is substantially reduced by mechanisms that remain unclear^[4,21]. A similar phenomenon, called the “macrophage disappearance reaction” occurs in other tissues

after injury^[22]. Although the importance of this to the pathogenesis of liver injury after APAP overdose is not known, KC numbers return to baseline levels by 72 h, through the local proliferation of mature KC^[4]. One intriguing mechanism by which KC “disappear” after APAP overdose may be through pyroptosis. Pyroptosis is a form of necrotic cell death that occurs in macrophages exposed to pathogens. This form of cell death produces macrophage lysis resulting in the release of high concentrations of proinflammatory cytokines. The importance of this process to KC disappearance and cytokine induction after APAP overdose, however, remains to be investigated.

FUNCTION OF MONOCYTE-DERIVED MACROPHAGES IN LIVER REPAIR AFTER APAP-INDUCED LIVER INJURY

Studies have shown that a population of monocyte-derived macrophages, distinct from KC and other resident macrophages, rapidly infiltrate the liver after APAP overdose^[23]. KC and monocyte-derived macrophages can be distinguished by flow cytometry based upon their level of expression of F4/80 and CD11b^[4,23]. In APAP-treated mice, KC are identified as a CD11b^{low} F4/80^{hi} population whereas monocyte-derived macrophages are identified as a CD11b^{hi} F4/80^{low} population that transiently appears in the liver 12 h after APAP challenge^[4,23]. These macrophages are likely distinct from resident monocyte-derived macrophages as they do not express Cx3cr1 at the onset of recruitment^[4].

Several studies have demonstrated that monocyte-derived macrophages are recruited to the liver after injury by the chemokine, chemokine (C-C motif) ligand 2 (Ccl2), also called monocyte chemoattractant protein-1. This chemokine stimulates chemotaxis of monocytes by activating the C-C chemokine receptor type 2 (Ccr2)^[24]. After APAP overdose, hepatic expression of Ccl2 is increased in hepatocytes and KC by 12 h after administration^[21]. This is soon followed by the accumulation of Ccr2-positive monocyte-derived macrophages. A role for Ccl2 in the recruitment of monocyte-derived macrophages to the liver after APAP overdose was confirmed by showing that monocyte-derived macrophage numbers were substantially reduced in the livers of Ccr2 knockout mice^[21,23]. Interestingly, although similar levels of injury were observed in wild-type and Ccr2 knockout mice following APAP challenge, there was a failure to clear necrotic cells from the livers, indicating an important role for monocyte-derived macrophages in the phagocytic removal of dead cells^[23]. Recently, it was reported that infusion of alternatively-activated macrophages into APAP treated mice enhances phagocytic clearance of dead cell debris, an approach that may be very valuable therapeutically in APAP overdose patients.

Further studies identifying macrophage subsets in the livers of mice following APAP challenge have demonstrated the dynamic presence of three distinct macrophage subsets^[4]. In these studies, Ly6C and the chemokine (C-X3-C motif) receptor 1 (Cx3cr1) were used to characterize different macrophage subsets. KC, which are Ly6C^{lo} Cx3cr1⁻, were significantly reduced at 24 h after APAP challenge (*i.e.*, macrophage disappearance reaction), while there was a dramatic increase in Ly6C^{hi} Cx3cr1⁺ macrophages that were recruited to the liver in a Ccr2- and M-CSF-dependent manner^[4]. By 72 h, the dominant macrophage population in the liver was Ly6C^{lo} Cx3cr1⁺, which was distinct from the KC population (Ly6C^{lo} Cx3cr1⁻). Adoptive transfer experiments using green fluorescent protein- (GFP)-labeled monocytes determined that the infiltrating Ly6C^{hi} Cx3cr1⁻ macrophages ultimately gave rise to the Ly6C^{lo} Cx3cr1⁺ macrophage subset^[4]. Molecular profiling using microarray analysis revealed that the Ly6C^{hi} Cx3cr1⁻ macrophages expressed high levels of proinflammatory genes, indicating an M1-like phenotype, while the Ly6C^{lo} Cx3cr1⁺ macrophages expressed high levels of pro-restorative and anti-inflammatory genes, indicating an M2-like phenotype^[4]. The gene expression profile of Ly6C^{lo} Cx3cr1⁺ macrophages was distinct from that of KC which demonstrated variable expression of pro-restorative genes. Collectively, these findings indicate that Ly6C^{hi} CX₃CR1⁻ proinflammatory macrophages rapidly accumulate in the liver after APAP overdose (Figure 1). These cells traffic into the necrotic foci where they phagocytose dead cell debris and switch phenotype to Ly6C^{lo} Cx3cr1⁺ pro-restorative macrophages. Consistent with this, it was recently reported that phagocytosis of neutrophils by macrophages triggers macrophage phenotype switching after APAP overdose. Once these cells switch phenotype, they produce pro-repair growth factors and anti-inflammatory cytokines that trigger the transition from the inflammatory phase of liver injury to the reparative phase. While the mechanism by which monocyte-derived macrophages are recruited to the liver is well established, the mechanisms controlling the intrahepatic trafficking, phagocytosis and phenotype switching by these cells remains poorly understood. Our recent studies, however, indicate that the enzyme

plasmin, a component of fibrinolysis, may be important for these processes.

REGULATION OF HEPATIC MACROPHAGE FUNCTION BY COMPONENTS OF FIBRINOLYSIS

Plasminogen, the zymogen form of the proteolytic enzyme plasmin, is a 90 kDa plasma glycoprotein that is produced in the liver and circulates in the blood^[25]. This protein is converted to its active form, plasmin, through proteolytic cleavage by either tissue-type plasminogen activator or urokinase-type plasminogen activator^[26]. Plasmin is a serine protease that is most well-known for its ability to degrade fibrin clots. Several other plasmin substrates have been identified, however, including coagulation proteins, components of the complement system, extracellular matrix proteins and several matrix metalloproteinases (for review, see^[27]). Similar to other proteases, such as thrombin, plasmin can activate intracellular signaling pathways through activation of one of several putative plasmin receptors^[28]. One of these receptors, annexin A2/S100A10, is a heterotetrameric complex composed of two molecules of annexin A2 and two molecules of S100A10. Studies have shown that plasmin stimulates production of proinflammatory cytokines by human monocyte-derived macrophages through this receptor by a mechanism that requires activation of mitogen-activated protein kinases and nuclear factor- κ B (NF- κ B)^[29,30]. Another putative plasmin receptor is the G protein-coupled receptor, protease-activated receptor-1 (PAR-1). PAR-1 activation by a variety of proteases produces a tethered ligand that binds to the receptor and activates signaling^[31,32]. Interestingly, treatment of mice with selective PAR-1 antagonists was shown to prevent plasmin-mediated migration of leukocytes into the pleural cavity, an effect that was dependent upon mitogen-activated protein kinases- and NF- κ B-dependent release of Ccl2^[33]. In addition to these receptors, several other putative plasmin receptors have been identified that stimulate signaling in macrophages, including enolase-1, histone H2B, and Plg-Rkt^[28,34-36].

Several studies indicate that plasmin is a key regulator of monocyte and macrophage function in the liver after injury. For example, plasminogen deficiency was shown to impair recruitment of macrophages to the liver after a stab injury^[37,38]. Others have demonstrated that phagocytic clearance of antibody labeled erythrocytes by KC was substantially reduced in plasminogen knockout mice indicating an important role for plasmin in regulation of phagocytosis^[39]. Consistent with this finding, Bezerra and colleagues demonstrated that deficiency in plasminogen prevented clearance of dead hepatocytes after treatment with a hepatotoxic dose of carbon tetrachloride^[40]. Because these studies indicate a key role for plasmin in regulation of hepatic macrophages, we recently evaluated the role of plasmin in regulation of macrophage function after APAP overdose.

In mice treated with APAP, plasmin activity is increased in the liver by 6 h after treatment^[41]. Interestingly, inhibition of plasmin activity with tranexamic acid prevents detachment of sinusoidal endothelial cells and subsequent sinusoidal hemorrhaging^[41]. To examine the impact of plasmin generation on macrophage activation, we similarly treated mice with APAP followed by treatment with tranexamic acid. These studies revealed that inhibition of plasmin activity prevented early upregulation of several proinflammatory cytokines, including TNF- α , Ccl2 and the neutrophil chemokines Cxcl1 and Cxcl2^[42]. Because KC contribute to early induction of proinflammatory cytokines, we evaluated whether plasmin directly stimulates these cells to produce cytokines^[42]. Similar to *in vivo*, treatment of these cells with plasmin increased expression of TNF- α , Ccl2, Cxcl1 and Cxcl2 consistent with the hypothesis that plasmin directly activates KC (Figure 2)^[42]. It has been proposed that the damage-associated molecular pattern molecule, high-mobility group B1 (HMGB1) protein is released from damaged hepatocytes and triggers KC cytokine release through a toll-like receptor 4- and receptor for advanced glycation end products-dependent mechanism^[43]. In support of this, hepatocyte-specific deletion of HMGB1 was shown to reduce cytokine release after APAP overdose^[43]. Surprisingly, though, we found that treatment of KC with recombinant HMGB1, at concentrations above those detected in the blood after APAP overdose, had no effect on proinflammatory cytokine synthesis in KC^[42]. Remarkably, though, HMGB1 synergistically enhanced upregulation of cytokines in these cells^[42]. This suggested that fibrinolysis, in the face of ongoing liver injury (*i.e.*, HMGB1 release) produces a more robust inflammatory response. Our studies showed further that upregulation of proinflammatory cytokines by plasmin, and the synergistic enhancement by HMGB1, occurred by an NF- κ B-dependent mechanism. Interestingly, though, unlike previous studies, upregulation of proinflammatory cytokines by plasmin did not require either

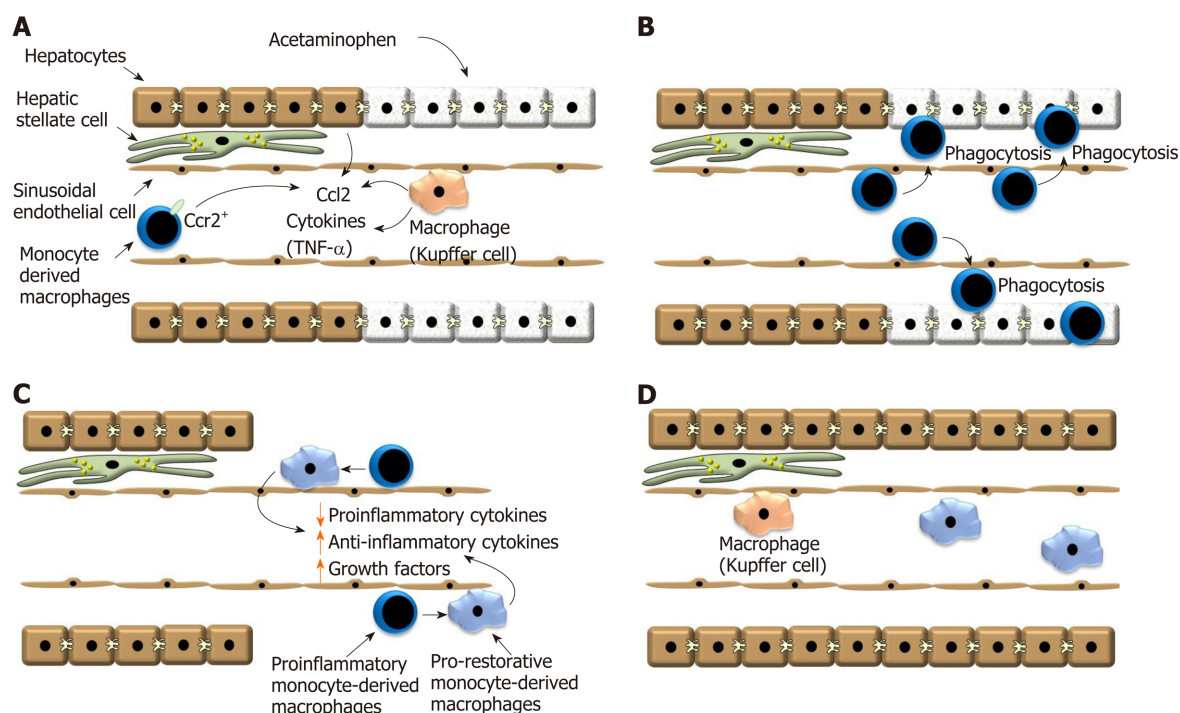


Figure 1 Ly6Chi CX3CR1- proinflammatory macrophages rapidly accumulate in the liver after acetaminophen overdose. **A:** After exposure to a hepatotoxicant, such as acetaminophen, hepatocyte necrosis triggers release of Ccl2 by hepatocytes and Kupffer cells. Ccl2 recruits Ccr2 expressing monocytes to the liver that ultimately become macrophages; **B:** Monocyte-derived macrophages traffic into the necrotic lesions where they phagocytose dead cell debris; **C:** Monocyte-derived macrophages then transition from a proinflammatory phenotype into a pro-reparative phenotype. This process decreases synthesis of proinflammatory cytokines, increases synthesis of anti-inflammatory cytokines and pro-reparative growth factors; **D:** Proliferation of hepatic cells ultimately results in the restoration of the hepatic structure. Ccl2: Chemokine, chemokine ligand 2; Ccr2: C-C chemokine receptor type 2; TNF- α : Tumor necrosis factor.

annexin A2 or PAR-1 suggesting that another plasmin receptor may be important for this process in liver^[42].

As discussed, after APAP-induced liver injury, monocyte-derived macrophages are recruited to the liver. These cells accumulate in the necrotic foci where they phagocytose dead cell debris^[44]. During this process, these cells switch phenotype from a proinflammatory macrophage to an anti-inflammatory, pro-restorative macrophage which decreases synthesis of proinflammatory cytokines and increases production of pro-repair growth factors^[41]. Paradoxically, whereas inhibition of plasmin prevented early cytokine induction in KC, it also prevented termination of cytokine synthesis at later times after APAP overdose^[42]. Further evaluation revealed that plasmin inhibition did not affect accumulation of monocyte-derived macrophages, however, it prevented trafficking of these cells into the necrotic lesions which prevented phagocytic removal of dead cells, similar to observations in carbon tetrachloride-treated mice (Figure 2)^[40,42]. This resulted in a failure of proinflammatory monocyte-derived macrophages to transition to pro-restorative macrophages leading to a persistence of proinflammatory cytokine production^[42]. While the mechanism by which plasmin stimulates monocyte-derived macrophage migration into necrotic foci remains unclear, it may have resulted from a failure to remove fibrin clots deposited in the lesions^[45]. A recent study showed that plasmin is required for migration of macrophages into the peritoneal cavity, and that plasmin facilitates macrophage migration by removing fibrin that impedes their movement^[46]. Further, plasmin can activate various matrix metalloproteinases, such as matrix metalloproteinase 9, that are critical for macrophage movement through extracellular matrix^[27]. While these are possibilities, this remains to be determined in the liver. What also remains to be determined is the mechanism by which plasmin promotes macrophage phenotype switching during liver repair. While plasmin directly stimulates proinflammatory cytokine release from KC, we found that it did not directly stimulate proinflammatory monocyte-derived macrophages to transition to pro-restorative macrophages (B.L.C., unpublished observation). This suggests that plasmin facilitates this process by an indirect mechanism. One possibility is the failed removal of dead cell debris by these cells when plasmin is inhibited. Phagocytosis is a well-known stimulus for the conversion of proinflammatory macrophages into pro-restorative macrophages and as discussed earlier, phagocytosis of neutrophils appears to be important for

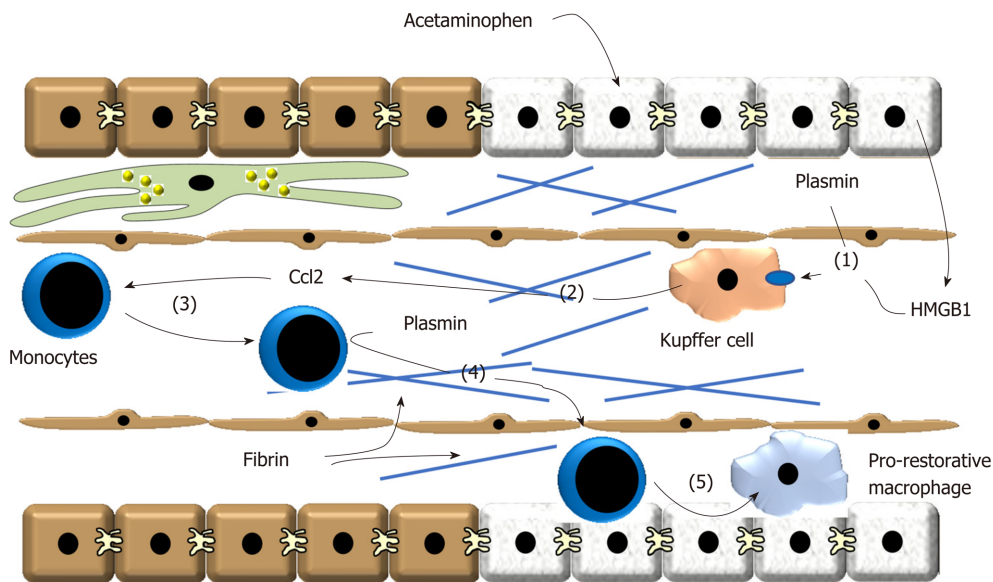


Figure 2 Treatment of these cells with plasmin increased expression of tumor necrosis factor- α , Ccl2, Cxcl1 and Cxcl2 consistent with the hypothesis that plasmin directly activates Kupffer cells. (1) After acetaminophen overdose, plasmin is generated and high-mobility group B1 is released from dead hepatocytes. These synergize to stimulate release of (2) pro-inflammatory cytokines, including Ccl2, from Kupffer cells. (3) Ccl2 stimulates recruitment of proinflammatory monocytes that accumulate at the periphery of the necrotic lesion. (4) Plasmin generation stimulates degradation of fibrin and/or activate matrix metalloproteinases that facilitate trafficking of the monocytes into the injured region. (5) The monocytes phagocytose dead cells which contributes to their conversion of pro-restorative macrophages. Ccl2: Chemokine, chemokine ligand 2; HMGB1: High-mobility group B1.

macrophage phenotype switching after APAP overdose^[47]. In further support of this, we found that *ex vivo* culture of monocyte-derived macrophages, isolated from the livers of APAP-treated mice, with necrotic hepatocytes terminates production of proinflammatory cytokines^[42]. Therefore, plasmin may facilitate the migration of monocyte-derived macrophages into the necrotic lesions, thereby putting them in close proximity to dead cells debris, the key stimulus for phenotype switching.

CONCLUSION

In summary, macrophages play a key role in the response to liver injury. Depending upon the macrophage type, they can produce proinflammatory cytokines (*i.e.*, KC), which recruit other immune cells, such as monocyte-derived macrophages that clear dead cell debris and terminate the proinflammatory response. A great deal remains to be determined regarding the mechanisms controlling these processes, however, and in particular, how plasmin contributes to their regulation. Elucidation of these mechanisms is important, because, studies have revealed that macrophage dysfunction is a key feature of ALF and that patients displaying features of macrophage dysfunction have the poorest outcome^[48,49]. It is possible that disruption of macrophage function impairs liver repair in a subset of patients ultimately leading to liver failure and a poor outcome. Interestingly, studies have shown that blood plasminogen levels are greatly reduced in patients with severe acute liver injury. While this remains to be determined, it is possible that this reduces plasmin activity in the liver thereby causing macrophage dysfunction and a poor reparative response in ALF.

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Sequencing of systemic treatment for hepatocellular carcinoma: Second line competitors

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Abstract

During the last decades, further knowledge of hepatocellular carcinoma (HCC) molecular mechanisms has led to development of effective systemic treatments including tyrosine kinase inhibitors (TKIs) and immunotherapy. In this review, we describe first and second line systemic treatment options for advanced HCC. Several trials have evaluated new drugs for the treatment of HCC patients: In first line, lenvatinib resulted non-inferior to sorafenib and it can be used as alternative, even in the lack of evidence for sequential treatment options in second line after lenvatinib. Recently, atezolizumab plus bevacizumab have shown superiority over sorafenib in first-line. Sorafenib-regorafenib sequential administration in selected patients has opened a new paradigm of treatment in advanced HCC with a life expectancy exceeding two years. Other TKIs for second line treatment include cabozantinib and ramucirumab (specifically for patients with Alpha-fetoprotein values ≥ 400 ng/mL). The combination of TKIs with immunotherapy may represent a big step forward for these patients in the near future.

Key words: Hepatocellular carcinoma; Systemic; Options; Sequencing; Advanced; Future

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Core tip: The prognosis of advanced hepatocellular carcinoma (HCC) has been improved in the last years due to new available drugs for first and second line systemic treatments. Recent improvements in HCC molecular mechanisms have led to development of effective systemic treatments including tyrosine kinase inhibitors and immunotherapy. In this review, we describe first and second line systemic treatment options for advanced

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INTRODUCTION

Primary liver cancer or hepatocellular carcinoma (HCC) is the fifth most common neoplasm and the third leading cause of cancer related death worldwide^[1]. Its prognosis in advanced stages has been improved in the last years because of new available drugs for first and second line systemic treatment options^[2].

During the last decades, further knowledge of HCC molecular mechanisms has led to the development of effective systemic treatment including tyrosine kinase inhibitors (TKIs) and immunotherapy. In this review, we describe first and second line systemic treatment options for advanced HCC focusing on sequencing therapy (sorafenib-based treatment) and comparison of different second line options (Figure 1).

FIRST LINE SYSTEMIC TREATMENT

Sorafenib was the first effective treatment approved for advanced HCC^[3]. Two double-blind, randomized clinical trials (RCTs) demonstrated a better overall survival (OS) with a relative risk reduction of death of 70% [HR (hazard ratio) of 0.69 [95% confidence interval (CI): 0.55; 0.87]] for sorafenib against placebo/best supportive care^[3,4]. Likewise, a benefit was observed in prolonging time to radiological progression (TTP), evaluated through Response Evaluation Criteria for Solid Tumors (RECIST 1.0)[HR: 0.58 (95% CI: 0.45; 0.74)]^[5,6]. In these trials, sorafenib could be continued even after radiological tumor progression, *i.e.*, until symptomatic progression, intolerance or death. The median TTP under sorafenib was 5.5 mo. In the SHARP and Asia-Pacific trials, although there was a low objective response rate (ORR) (less than 2%), stable disease (SD) was observed in more than 70% with a disease control rate (DCR) of 43% and 53%, respectively^[3,4]. Thus, sorafenib reached its main primary end-point with a benefit in overall survival, even without significant tumor shrinkage. This phenomenon opened a paradigm in clinical oncology and trial design for systemic treatment for HCC.

Several observational cohort studies validated the use of sorafenib in the real life setting. The GIDEON study was an observational prospective cohort study whose primary objective was to evaluate the effectiveness of sorafenib in these scenarios, particularly in patients with advanced liver disease^[7]. There was a higher incidence of adverse events (AEs) and treatment discontinuation due to AEs in patients with Child Pugh B-C when compared to Child Pugh A. Median survival was significantly lower in patients with unpreserved liver function. Other series from Italy and Argentina reported similar outcomes^[8,9].

Some authors still argue that systemic chemotherapy has a therapeutic role in these patients since sorafenib showed a low radiological response, a poor gain in survival, absence of predictive response factors and a high cost. However, over the past decades, trials or uncontrolled interventional studies with doxorubicin^[10-13], cisplatin, oxaliplatin or FOLFOX, gemcitabine-based GEMOX^[14-19], capecitabine-based XELOX^[20] or in combination with bevacizumab^[21], have all failed with no proven efficacy and eventually accompanied by high toxicity rates. Finally, recent trials exploring the efficacy of sorafenib in combination with arterial chemotherapy have shown contradictory results^[22,23].

Since 2008, different molecular pathways of HCC development and progression have been studied in depth. Based on these studies, many clinical trials were conducted testing new drugs for HCC first-line treatment. To date only lenvatinib has demonstrated to be non-inferior to sorafenib (Table 1). Sunitinib, an endothelial growth factor inhibitor (EGFR), has failed to demonstrate non-inferiority in a phase III RCT^[24]. There was a lot of expectation with brivanib (BRISK-FL trial) given its anti-angiogenic effect and different inhibition of pathways than sorafenib (vascular-

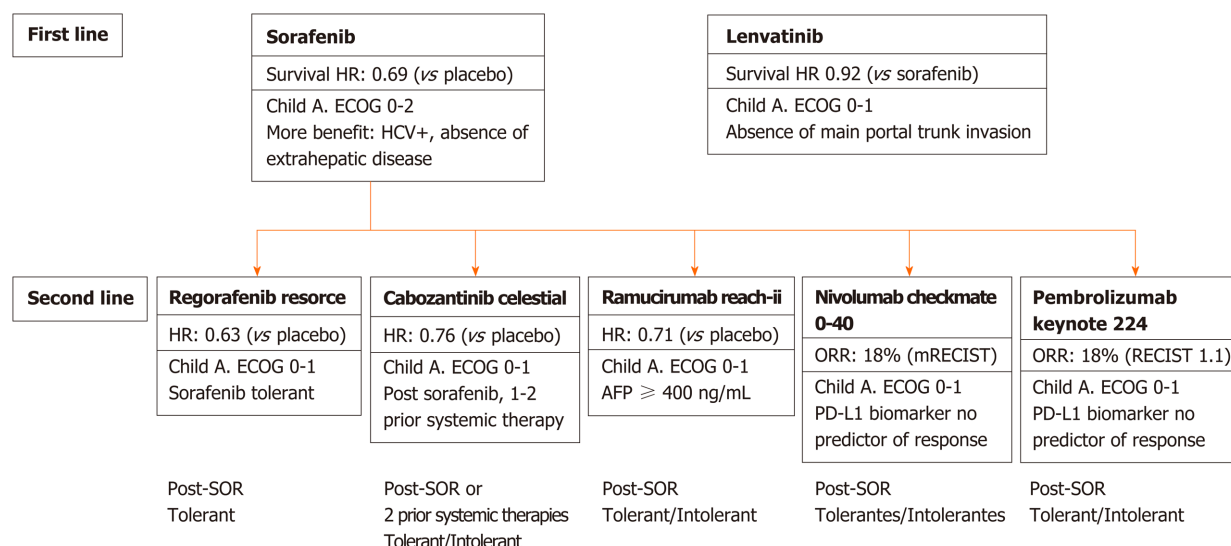


Figure 1 First and second line therapies for advanced hepatocellular carcinoma. RECIST: Response Evaluation Criteria for Solid Tumors; ECOG: Eastern Cooperative Oncology Group; SOR: Sorafenib.

endothelial growth factor -VEGF- and fibroblast growth factor-FGF-pathways). However, it was also not efficient in a non-inferiority trial^[25]. Other phase III trials failed to show superiority, including: erlotinib + sorafenib *vs* sorafenib + placebo (SEARCH trial, anti EGFR)^[26], linifanib *vs* sorafenib (VEGF and platelet-derived growth factor inhibitor-PDGF)^[27]. Likewise, dovitinib (VEGF, FGF and PDGF inhibitor)^[28] and bevacizumab (anti-VEGF monoclonal antibody) did not demonstrate efficacy in phase II trials, presenting excessive toxicity and high incidence of sepsis, not allowing for further studies^[29].

The only pre-treatment sorafenib predictors of better survival are the absence of extrahepatic disease, hepatitis C as an underlying disease and a low neutrophil/leukocyte ratio^[30]. High serum Alpha-fetoprotein (AFP) values (> 200 ng/mL) and macroscopic vascular invasion are baseline variables associated with poor prognosis in these patients, but even in these subgroups, sorafenib showed a survival benefit *vs* placebo^[30].

Results of a phase II and then a phase III RCT (REFLECT trial), have shown that lenvatinib, a VEGF receptors 1-3, FGF receptors 1-4 and PDGF α receptor inhibitor, was the first agent achieving non-inferiority against sorafenib^[31,32]. The eligibility criteria in the REFLECT study were different from SHARP and Asia-Pacific studies, *i.e.*, excluding those patients with main portal trunk tumor invasion and those subjects with intrahepatic tumor involvement of more than 50% of total liver volume^[32]. The REFLECT study was an open-labeled phase 3 RCT, in which the primary objective was non-inferiority survival with respect to sorafenib (upper HR confidence interval limit of 1.08)^[32]. In the intention-to-treat analysis, patients with lenvatinib presented a median survival of 13.6 mo, whereas those in the sorafenib arm had a median survival of 12.3 mo (HR: 0.92, 95% CI: 0.79-1.06). Likewise, a better progression-free survival (PFS) and TTP were observed, together with a higher ORR for lenvatinib over sorafenib (Figure 2).

Although this study demonstrated that lenvatinib is an effective first-line treatment option for advanced HCC, there are some important points to be considered. First, the non-inferiority design should have been characterized by less toxicity rates with lower discontinuation rates due to AEs as a co-primary end-point. However, these events were not included as co-primary end-points^[32]. Second, the non-blinded design might have generated a possible intervention bias, justifying the longer treatment duration in lenvatinib arm (median treatment duration of 5.7 mo) compared to sorafenib arm (median treatment duration of 3.7 mo)^[32]. Thus, if there was a similar tolerance between groups, this observation is striking and can only be explained by the design. Treatment duration with sorafenib was shorter, even when compared with previous RCT^[34] and there was not a significant difference regarding treatment discontinuation rates between both arms. Consequently, the effect upon survival might have been biased due to a premature sorafenib interruption. Indeed, a higher incidence of severe AEs were observed in the lenvatinib arm (57% *vs* 49%)^[32]. Lenvatinib was characterized by a higher incidence of arterial hypertension, proteinuria, dysphonia and hypothyroidism, while diarrhea, hand-foot reaction and alopecia were more

Table 1 First line agents failed for the treatment of advanced hepatocellular carcinoma

Study drug	Population	Design-intervention	Results
Sunitinib (EGFR), Cheng <i>et al</i> ^[24] , 2013	n = 1074, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase III. Non-inferiority. Sunitinib <i>vs</i> Sorafenib	Failed to reach its primary end-point. Higher rate of EAs
Brivanib (VEGF, FGF), Johnson <i>et al</i> ^[25] , 2013	n = 1150, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase III. Non-inferiority. Brivanib <i>vs</i> Sorafenib (Bristol)	Failed to reach its primary end-point. Higher rate of EAs
Erlotinib (EGFR), Zhu <i>et al</i> ^[14] , 2006	n = 720, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase III. Superiority, Erlotinib + Sorafenib <i>vs</i> Placebo + Sorafenib	OS similar, TTP similar, Similar EAs
Linifanib (VEGF, PDGF), Cainap <i>et al</i> ^[27] , 2015	n = 1035, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase III. Superiority, Linifanib <i>vs</i> Sorafenib	Failed to reach its primary end-point. TTP better for linifanib, Similar EAs
Tigatuzumab, Bruix <i>et al</i> ^[30] , 2017	n = 162, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase II, Tigatuzumab + Sorafenib <i>vs</i> Placebo + Soraf	Safety profile adequate but no better TTP and OS
Dovitinib (VEGF, FGF, PDGF), Cheng <i>et al</i> ^[28] , 2016	n = 165, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase II. Dovitinib <i>vs</i> Sorafenib	OS non superior, TTP similar, Higher rate of EAs
Bevacizumab (Ab VEGF), Hubbard <i>et al</i> ^[29] , 2016	n = 17, BCLC B-C, ECOG 0-1, Child Pugh A/B	RCT Fase I/II, Bevacizumab + Sorafenib	Higher rate of EAs, Excessive toxicity

BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group; EGFR: Endothelial growth factor; FGF: Fibroblast growth factor; OS: Overall survival; PDGF: Platelet-derived growth factor inhibitor; TTP: Time to progression; VEGF: Vascular-endothelial growth factor; RCT: Randomized clinical trials.

frequent with sorafenib. However, this is opposed to the fact that lenvatinib showed higher tumor shrinkage rates^[32]. Moreover, the adoption of different second line drugs (that subsequently revealed to be effective) following sorafenib and lenvatinib, might have influenced the post-progression overall survival.

The REFLECT trial modifies the future therapeutic options in patients with advanced HCC. It remains unclear which subgroup of patients will benefit more with one drug or another, as well as what will be the drug of choice for second line after tumor progression with lenvatinib. Thus, the appropriate selection of each treatment should be individualized.

More recently, immunotherapy has evolved as a potential first line systemic option. From a previous phase Ib-II trial escalating-dose, nivolumab (3 mg/kg every 2 wk-schedule) showed promising tumor responses in sorafenib-experienced patients^[33]. These results led to perform a phase III RCT, in which nivolumab was tested against sorafenib in the first-line setting (Check-Mate 459 study; NCT02576509). Unfortunately, results were negative for both co-primary end-points of OS [16.4 mo (95%CI: 13.9-18.4) *vs* 14.7 mo (95%CI: 11.9-17.2), $P = 0.0752$] and PFS [3.7 mo (95%CI: 3.1-3.9) *vs* 3.8 mo (95%CI: 3.7-4.5)].

These negative results have been recently counterbalanced by positive results of a phase III, open-label, randomized trial evaluating the combination of atezolizumab, another immune-checkpoint inhibitor, with bevacizumab, an anti-VEGF monoclonal antibody, compared to sorafenib. Eligibility criteria included preserved liver function, advanced HCC, ECOG 0-1 in the absence of main portal trunk invasion and immunological disorders. Both co-primary end-points, OS HR 0.58 (CI: 0.42-0.79) [median survival not reached *vs* 13.2 mo with sorafenib alone, $P = 0.0006$] and PFS HR 0.59 (CI: 0.47-0.76; $P < 0.0001$) were longer for the new treatment combination (NCT03434379; IMbrave150 study). A significant higher ORR rate in the combination arm 27% *vs* 12% and DCR of 74% *vs* 55% ($P < 0.0001$) were observed. Similar incidence of all grade adverse events and a lower incidence of grades 3/4 related adverse events were observed with the combination arm (36% *vs* 46%). The most frequent adverse events were systemic hypertension, diarrhea, proteinuria, hyporexia, elevated liver enzymes and infusional reaction. However, treatment discontinuation was higher in atezolizumab + bevacizumab arm (16% and 10%, respectively) These results opened a new and potentially unlimited therapeutic options and are currently being studied in several phase-3 trials.

SECOND LINE SYSTEMIC TREATMENT: WHEN AND TO WHOM?

Three potential scenarios can develop during first line systemic treatment, which determine the subsequent patients' management: (1) Tolerance or intolerance; (2) Radiological progression; and (3) Symptomatic progression (Figure 3).

Tolerance has been defined and applied as eligibility criteria in second line phase

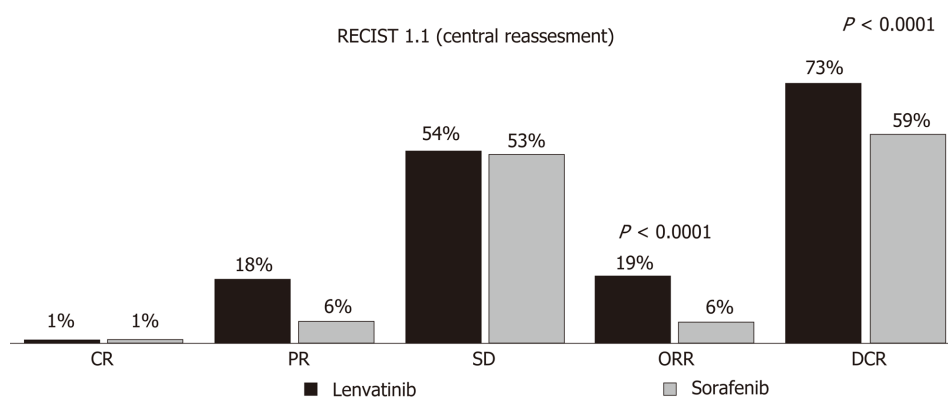


Figure 2 Radiological tumor response between sorafenib and lenvatinib according to RECIST 1.1 criteria, reported in the REFLECT trial. RECIST: Response Evaluation Criteria for Solid Tumors; ORR: Objective response rate.

III RCTs after sorafenib treatment. In the RESORCE trial, tolerance was defined according to a specific time-dosing schedule as ≥ 400 mg during at least 20 d of the last 28 d under sorafenib^[34]. In another trial, this definition was focused on the severity of adverse events, its complete resolution and no recurrence after reintroduction of sorafenib^[35]. Consequently, tolerance or intolerance have been differently defined in RCT, focusing on the dosing-scheme or based on AEs severity. These clinical definitions are important to systematize the decision-making process in the daily practice.

The concept of radiological tumor progression has been focused during the last decade. As previously discussed, sorafenib granted an OS benefit even without tumor shrinkage^[3,4]. On the other hand, TTP has failed to be a surrogate marker of survival benefit in HCC. For this reason, the reliability of TTP as a survival predictor is questionable in HCC, eventually other measures like disease free survival should be taken into account. Several examples have been published showing a benefit in TTP without any significant improvement in OS^[36,37]. Thus, it seems that TTP does not accurately correlate with OS in advanced HCC. TTP might be exposed to several bias. Tumor progression can be evaluated by RECIST 1.1 or modified RECIST criteria for HCC (mRECIST)^[38]. It should be noted that there might be some inconsistencies defining new lesions between both criteria. Moreover, RECIST 1.1 is more stringent than mRECIST when defining partial and complete responses and mRECIST could be more difficult to assess under heterogeneous tumor enhancement areas.

Radiological tumor defines eligibility for second-line treatment. However, radiological tumor progression can be distinguished in four different patterns with a different impact on OS^[39,40]. The key clinical question is when to move to second line treatment under tumor progression in a patient tolerating treatment or even more strikingly, if during treatment the patient has shown clinical benefit hallmarks such as dermatological events^[41]. A new intrahepatic lesion has a better prognosis than a new extrahepatic lesion or a new vascular invasion^[39,40]. Therefore, second-line systemic treatment might be initiated under worst types of patterns of progression and may be delayed for a second progression in case of intrahepatic progression pattern.

Symptomatic progression is defined as progression beyond an ECOG PS 2^[42]. Since most HCCs develop in patients with chronic liver disease, the conjunction of tolerability, treatment complications and risk of cirrhosis decompensation, make this cancer a huge therapeutic challenge. In the same patient, symptomatic progression might be due to cancer-related symptoms or those associated with liver disease decompensation. Therefore, in the same patient there may be “tumor radiological progression” or “untreatable progression” due to development of liver decompensation as a competing event for OS^[40].

Following systemic treatment, drug-related adverse events and complications due to liver disease should be evaluated at each visit, which will determine the need for dose reduction, transient or definitive suspension of the drug. These events define a patient as “intolerant” or “not a candidate” for second line treatment at all. This definition is important in the clinical practice since it defines not only who is potential candidate to start treatment with second-line drugs but also with which drug may be treated.

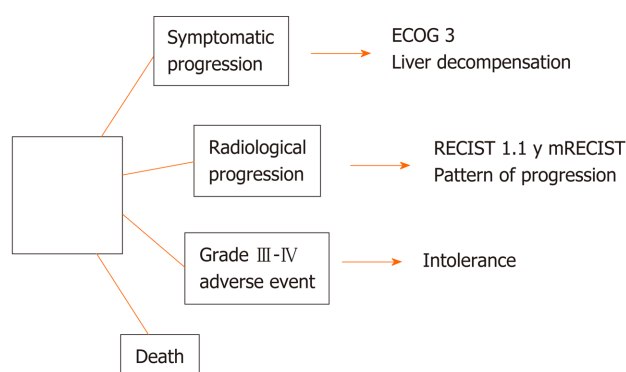


Figure 3 Clinical “stopping rules” of first and second line tyrosine kinase inhibitors. RECIST: Response Evaluation Criteria for Solid Tumors; ECOG: Eastern Cooperative Oncology Group; RECIST: Response Evaluation Criteria for Solid Tumors criteria.

SECOND LINE COMPETITORS

Several clinical trials failed to show any benefit as 2nd line treatment option for advanced HCC in terms of OS when compared to placebo until the RESORCE study^[34]. Brivanib (BRISK-PS, phase III RCT)^[36], axitinib (phase II study)^[43], everolimus (EVOLVE-1 phase III RCT)^[44] and tivantinib^[45] have all failed to show a benefit in OS *vs* placebo (Table 2). In particular, Tivantinib, a MET pathway inhibitor (hepatocyte growth factor blockade), had shown promising results in a phase II RCT^[46]. However, a phase III study paradoxically turned negative, even in patients highly expressing MET mutation^[45]. This RCT has shown the lack of utility of tumor biopsy as a predictor of the therapeutic response in HCC (c-MET), subsequently confirmed in the CHECKMATE 040 and KEYNOTE-240 studies with nivolumab and pembrolizumab^[33,35], where the expression on PD-1 in tumor tissue failed to predict treatment response.

Currently, regorafenib^[34], cabozantinib (CELESTIAL phase III RCT)^[47] and ramucirumab (REACH I and REACH II phase III RCTs)^[48] have demonstrated second-line efficacy. A controversial issue has raised with pembrolizumab regarding statistical and clinical results of the KEYNOTE-224 RCT. Although the trial was negative according to its pre-established primary efficacy end-points, it showed a survival benefit that promoted its acceptance as a new second line systemic option^[35]. On the other hand, nivolumab can be used as a second line option—at least in United States, following positive results of the uncontrolled phase I/II CHECK MATE 040 trial^[33].

ANTI-TYROSIN KINASE INHIBITORS FOR SECOND LINE THERAPY

Both sorafenib and regorafenib are TKIs, but regorafenib is more active on VEGF, produces more intense inhibition on c-KIT receptor and partially blocks the TIE2 receptor^[49]. The RESORCE phase III RCT included patients with advanced HCC who were tolerant and progressed under treatment with sorafenib^[34]. The randomization was stratified 2:1 according to AFP values > 400 ng/mL, presence of macrovascular invasion, extrahepatic spread and ECOG 0-1. The median OS was 10.6 mo (CI: 9.1; 12.1) for regorafenib and 7.8 mo (CI 6.3; 8.8) for placebo, with a HR of 0.62 (95%CI: 0.50-0.79)^[34]. Likewise, regorafenib presented a benefit over TTP, with an ORR of 11% and a DCR of 65%; evaluated through both RECIST 1.1 and mRECIST. In post-hoc analyzes, regorafenib presented an OS benefit in all clinical scenarios including patients with baseline worst prognosis^[34]. Overall, 93% of the patients receiving regorafenib presented AEs (*i.e.*, high blood pressure, fatigue, diarrhea and hand-foot reaction), 46% grade III and 4% grade IV, with drug discontinuation due to intolerance in 10% of the patients^[34] (Table 3).

Cabozantinib has been recently approved by both EMA and FDA as another second line treatment option. The phase III CELESTIAL RCT of cabozantinib 60 mg/d showed positive results *vs* placebo for OS [HR: 0.76 (CI: 0.63; 0.93); *P* = 0.005] and disease free survival [HR: 0.44 (CI: 0.36; 0.52)]^[47]. Cabozantinib, initially considered a dual VEGFR-2 and c-MET inhibitor, subsequently showed activity on MET, AXL,

Table 2 Second line tyrosine kinase inhibitors approved for the treatment of advanced hepatocellular carcinoma

Study drug	Population	Design-intervention	Results
Regorafenib, Bruix <i>et al</i> ^[34] , 2017, resorice	<i>n</i> = 553, BCLC B-C, ECOG 0-1, Child Pugh A, SOR-Tolerant	RCT phase III. Superiority. Regorafenib <i>vs</i> placebo	OS HR 0.63 (CI: 0.50-0.79), ORR 11%, DCR 65% (mRECIST)
Cabozantinib, Abou-Alfa <i>et al</i> ^[47] , Celestial	<i>n</i> = 707, BCLC B-C, ECOG 0-1, Child Pugh A, 1-2 prior systemic treatment, SOR-Tolerant/intolerant	RCT phase III. Superiority. Cabozantinib <i>vs</i> placebo	OS HR 0.76 (CI: 0.63-0.93), ORR 4%, DCR 64% (RECIST 1.1)
Ramucirumab, Zhu <i>et al</i> ^[48] , reach I-II	<i>n</i> = 542, BCLC B-C, ECOG 0-1, Child Pugh A, AFP ≥ 400 ng/mL, SOR-Tolerant/intolerant	RCT phase III. Superiority. Ramucirumab <i>vs</i> placebo	OS HR 0.69 (CI: 0.57-0.84), ORR 5%, DCR 60%, (RECIST 1.1)

Comparison across studies should cautiously analyzed. BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group; OS: Overall survival; HR: Hazard ratio; SOR: Sorafenib; ORR: Objective response rate; RCT: Randomized clinical trials; DCR: Disease control rate; AFP: Alpha-fetoprotein.

RET, FLT3 and Tie-2 pathways^[50]. The CELESTIAL study included patients with advanced HCC, Child Pugh A, ECOG PS 0-1, with up to two previous systemic treatments, including sorafenib prior exposure, independently from tolerance. Patients were stratified according to etiology of liver disease (HBV or HCV), presence of macrovascular invasion, extrahepatic spread and world region and were randomized 2:1. The OS was 10.2 mo (CI: 9.1-12.0) for cabozantinib and 8 mo (CI: 6.8-9.4) for placebo. Cabozantinib presented lower TTP, with an ORR of 4%, SD of 60% and a DCR of 64%^[47]. Dose reductions and discontinuations were more common in the cabozantinib arm, as were AEs (Table 3). No data have been reported in the post-hoc analysis regarding the effect of cabozantinib according to sorafenib tolerance, while cabozantinib showed higher survival than placebo with a median survival of 11.3 mo *vs* 7.2 mo [HR: 0.74 (CI: 0.59-0.92)] among patients receiving sorafenib as the single prior systemic therapy^[47].

OTHER ANTIANGIOGENIC AGENTS FOR SECOND LINE THERAPY

Prior studies have shown that AFP values are associated with worst OS in patients with advanced HCC and correlate with VEGF pathways, a critical role in angiogenesis. The anti-VEGF monoclonal antibody, ramucirumab, has been initially tested in second line treatment (whether sorafenib tolerant or not) in two phase III RCTs^[48,51]. In the REACH I study, there was not a significant difference in OS^[51]. However, from a post hoc analysis, ramucirumab showed better OS when compared to placebo in patients with AFP values equal or higher than 400 ng/mL^[51]. This led to the design of the REACH II trial, which included patients with advanced HCC with AFP values ≥ 400 ng/mL who were intolerant or progressed under sorafenib^[48]. This study confirmed that ramucirumab reduced the risk of death by 29%, with a median OS of 8.5 mo *vs* 7.3 mo for the placebo group [HR of 0.71 (95%CI: 0.53-0.95)]. Moreover, a better TTP was observed with an ORR and DCR of 5% and 60%, respectively. Ramucirumab was associated with a higher incidence of AEs, mainly hyponatremia and arterial hypertension (Table 3). This study was the first one showing efficacy based on a specific biomarker.

IMMUNOTHERAPY IN SECOND LINE THERAPY

Immunotherapy in HCC has been initially explored as second line options in patients with post-sorafenib tumor progression (tolerant or intolerant). Tremelimumab (anti-CTLA4) has been explored in an uncontrolled phase II exploratory trial in HCV+ patients following at least one prior systemic treatment^[52]. The CheckMate 040 study, a phase I/II uncontrolled trial, evaluated nivolumab (anti PD-1) dose expansion and escalation scheme in patients with advanced HCC, Child Pugh A or B^[33]. There was a promising ORR of 20%, with 3 complete responses (CR) and a DCR of 64%. The 9-mo survival rate was 74% (CI: 67%-79%). Baseline tumor levels of PD-L1 expression did not impact overall responses. The most common adverse effects observed were rash, elevation of liver and pancreatic enzymes and pruritus. Immunological adverse events were reported in less than 10% of the patients. This led to its temporary approval by the FDA in the United States as a second-line treatment option.

Table 3 Scheme dose, adverse events and discontinuation rate of first and second line tyrosine kinase inhibitors and anti-vascular-endothelial growth factor agents approved for the treatment of advanced hepatocellular carcinoma

Study drug	Dose reduction - interruption	Discontinuation rate
Sorafenib	26% dose reduction (any AE), 44% drug interruption (any AE), most frequent AE leading to dose reductions: diarrhea, hand-foot skin reaction and rash	11%
Lenvatinib	37% dose reduction (related-AE), 40% drug interruption (related-AE), Most frequent AE leading to dose reductions: not reported	9%
Regorafenib	68% dose reduction or drug interruption (any AE), most frequent AE leading to dose reductions: diarrhea, hand-foot skin reaction	10%
Cabozantinib	62% dose reduction or drug interruption (any AE), most frequent AE leading to dose reductions: diarrhea, hand-foot skin reaction	16%
Ramucirumab	34% dose reduction or drug interruption (any AE), most frequent AE leading to dose reductions: fatigue, peripheral edema, hypertension and anorexia	11%

Comparison across studies should cautiously analyzed. AE: Adverse event.

Finally, the KEYNOTE-224 (NCT02702401) phase III RCT compared the anti-PD1 pembrolizumab *vs* placebo for patients with advanced HCC, with tumor progression or intolerant to prior treatment with sorafenib^[35]. Included patients had advanced HCC without main portal trunk tumor invasion, ECOG PS 0-1 and preserved liver function and were stratified according to baseline AFP values > 200 ng/mL, ECOG 0-1 and world region. The trial did not meet its primary efficacy end-points (OS and PFS). However, median OS was longer for pembrolizumab (13.9 mo) *vs* placebo (10.6 mo) with a HR of 0.78 (CI: 0.61-0.99); $P = 0.024$. The upper limit of the CI almost crossed the line of no effect as proposed by the null hypothesis with an expected statistical significance of $P = 0.017$. Thus, this was the reason for this negative trial, although there was a higher ORR (18.8% *vs* 4.4%) and DCR (62% *vs* 53%). Most common treatment-related AEs were pruritus in 13%, fatigue 10%, increased liver function tests 9%, diarrhea 8% and rash 8%. Immune-mediated adverse events were reported in 18% of the patients with pembrolizumab, 7.2% being grade 3 or 4. However, OS was longer in pembrolizumab arm *vs* placebo when survival was adjusted for subsequent anticancer therapies (13.9 *vs* 9.3 mo; HR: 0.67; CI: 0.48-0.92; $P = 0.0066$) or a two-stage survival analysis model (10.6 *vs* 7.6 mo; HR: 0.68; CI: 0.53-0.86; $P = 0.0011$).

FIRST AND SECOND LINE SEQUENTIAL TREATMENT

A post-hoc retrospective analysis of the RESORCE trial evaluated the effect on survival of the sequential treatment of first and second line treatment with sorafenib-regorafenib: The sequential treatment granted 26 mo of median overall survival compared to 19.2 mo for the patients treated by sorafenib and placebo thereafter^[53]. Sixty percent of the study population had a prior last sorafenib dose of 800 mg/d. Regorafenib was effective regardless the last treatment dose of sorafenib (full dose *vs* lower dose) but patients with lower doses of sorafenib presented higher rates of hand-foot skin reaction, fatigue and anorexia when compared to placebo. Thus, caution should be taken when treating patients who were tolerant to lower doses of sorafenib after initiation of regorafenib.

Other data regarding sequential treatment of sorafenib-other TKIs are lacking, however data on sequential use of lenvatinib and sorafenib will be soon available by the post-hoc analysis of the REFLECT trial in those patients treated with sorafenib after lenvatinib discontinuation (Figure 4).

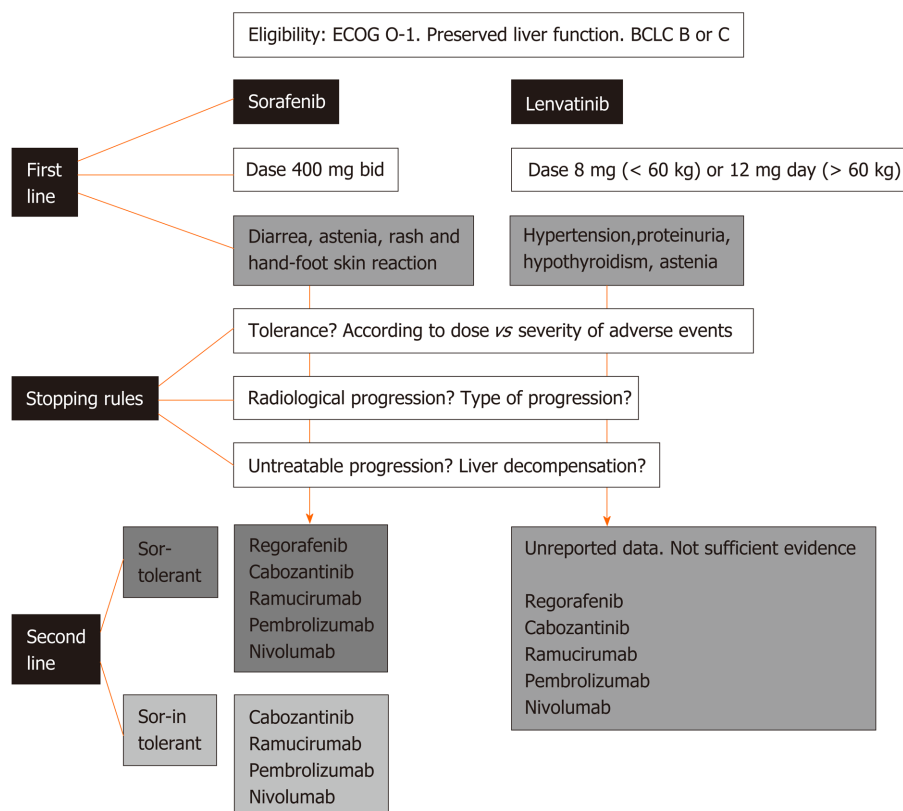


Figure 4 Flow chart for clinical-decision making processes of first and second line systemic treatment for advanced hepatocellular carcinoma. BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group.

SEQUENTIAL SYSTEMIC TREATMENT IN SPECIAL POPULATIONS

A special population excluded from RCT are patients with recurrent HCC following liver transplantation. The effect of sorafenib was reported in retrospective cohort studies with similar outcomes regarding survival and tolerability^[54]. More recently, a retrospective cohort study including 28 patients evaluated the sequential therapy of sorafenib-regorafenib in this setting^[55]. During regorafenib all patients had at least one adverse event, the most common grade 3/4 adverse events were fatigue and hand-foot skin reaction. Interaction between CYP3A4 metabolism was reported with higher plasma levels of immunosuppressive drugs increased. Median OS from regorafenib initiation was 12.9 (CI: 6.7-19.1 mo) and 38.4 mo (CI: 18.5-58.4 mo) from sorafenib initiation.

CONCLUSION

Atezolizumab plus bevacizumab may be the future standard of care over in first-line. However, some patients may be still be treated with with sorafenib or lenvatinib, particularly those patients with immunotherapy contraindication or main portal trunk invasion (not for lenvatinib). Both are equivalent, except for the orphan-based evidence of sequential post-lenvatinib treatment for second line. Sorafenib-regorafenib sequencing therapy has opened a new paradigm with a life expectancy exceeding two years at least for those patients tolerant for sorafenib. This data being previously unthinkable 10 years ago. Other therapeutic options for second line treatment include cabozantinib (for both sorafenib-tolerant and intolerant patients) and ramucirumab (only for patients with AFP values ≥ 400 ng/mL). While some regulatory agencies have approved the use of immunotherapy even after failing trials (*i.e.*, nivolumab and pembrolizumab), the identification of patients who could benefit from one or another option is still unclear. Other trials, either in first and second lines are being tested, with combination of immunotherapy plus TKIs, showing positive preliminary results. Further predictive biomarkers of treatment response are needed

in order to better select patients for each specific treatment.

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Therapeutic advances in non-alcoholic fatty liver disease: A microbiota-centered view

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent metabolic disorder with steadily increasing incidence rates worldwide, especially in the West. There are no drugs available at present to treat NAFLD, and the primary therapeutic options include weight loss and the combination of healthy diet and exercise. Therefore, novel interventions are required that can target the underlying risk factors. Gut microbiota is an "invisible organ" of the human body and vital for normal metabolism and immuno-modulation. The number and diversity of microbes differ across the gastrointestinal tract from the mouth to the anus, and is most abundant in the intestine. Since dysregulated gut microbiota is an underlying pathological factor of NAFLD, it is a viable therapeutic target that can be modulated by antibiotics, probiotics, prebiotics, synbiotics, fecal microbiota transplantation, and microbial metabolites. In this review, we summarize the most recent advances in gut microbiota-targeted therapies against NAFLD in clinical and experimental studies, and critically evaluate novel targets and strategies for treating NAFLD.

Key words: Non-alcoholic fatty liver disease; Gut microbiota; Probiotics; Prebiotics; Fecal microbiota transplantation; Metabolites

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Core tip: Non-alcoholic fatty liver disease is a highly prevalent metabolic disease worldwide. In this review, we summarize the most recent advances in gut microbiota-targeted therapies against non-alcoholic fatty liver disease, including antibiotics, probiotics, prebiotics, synbiotics, fecal microbiota transplantation, and the gut

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microbiota-derived components and metabolites.

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INTRODUCTION

The liver is the largest organ in our body, with vital functions in digestion, energy storage, and detoxification. Fatty liver disease is the most common hepato-pathological condition characterized by excessive fat accumulation in the liver. Based on the etiology, fatty liver disease can be classified into the alcoholic and non-alcoholic types. As the name indicates, alcoholic fatty liver disease is the result of alcohol overconsumption. Ethanol metabolism in the liver produces fatty acids which steadily accumulate within the liver cells, along with acetaldehyde and free radicals that also have deleterious effects on the liver and other organs^[1]. Non-alcoholic fatty liver disease (NAFLD) is caused by multiple factors including poor diet, insulin resistance, and other metabolic disturbances^[2]. In addition, lifestyle-related factors like sleep shortage, irregular food intake, sedentary habits, and excessive weight gain are also risk factors for NAFLD^[3]. It can be further sub-divided into the fatty liver without inflammation and nonalcoholic steatohepatitis (NASH) types. The latter frequently progresses to fibrosis, advanced cirrhosis, hepatocellular carcinoma (HCC), and even death^[4]. NAFLD is in fact the most rapidly increasing underlying condition requiring liver transplantation^[5], and can be considered a hepatic manifestation of the metabolic syndrome. Several clinical trials are underway to develop novel therapies against NAFLD^[6], since the current treatments focusing on lifestyle modifications have been largely approved. Mediterranean diet and physical activity for instance have been shown to prevent the onset of NAFLD^[7,8]. The ultimate goal of NAFLD treatment is to inhibit fibrotic development that can eventually lead to cirrhosis and HCC^[6]. However, there are no Food and Drug Administration approved drugs at present for treating this condition^[9].

The gut microbiota is considered by many as a “metabolic organ” that plays a vital role in host metabolism and liver function^[10]. In addition to the classic “two-hit” theory or the updated “multiple hit” model^[11], intestinal dysbiosis is also a causative factor of NAFLD, and promotes its progression by modulating host energy metabolism, insulin sensitivity, immune response, and inflammation^[12]. The pathophysiological relationship between the gut microbiota and NAFLD is complex and involves diverse immunological and metabolic pathways. For instance, impaired intestinal permeability in mice lacking the junctional adhesion molecule A protein (Jam1) or Muc2 increases the risk of liver inflammation when the animals are fed a high-fat diet (HFD)^[13,14]. Furthermore, the microbiota from adult NAFLD patients exhibits differences in carbon and amino acid metabolism^[15]. NAFLD is also associated with increased serum TMAO levels and hepatic bile acid (BA) synthesis^[16] and less production of phosphatidylcholine^[17]. The pathological roles of various bacterial metabolites and microbiota-generated secondary BA in NAFLD have been unearthed in recent years^[18]. These metabolites can trigger metabolic dysfunction and contribute to NAFLD development and progression by targeting relevant pathways. The gut microbiota also diversifies the repertoire of host BAs by modulating its metabolism, thereby regulating pathways mediated by BA receptors such as farnesoid X receptor (FXR) and TGR5^[19,20].

Therefore, researchers are increasingly focusing on the gut microbiota as a new therapeutic target for NAFLD, and have developed various treatment modalities including antibiotics, probiotics, prebiotics, synbiotics, fecal microbiota transplantation (FMT), gut microbiota-derived components, and metabolites (Figure 1). In this review, we summarize the most recent advances in gut microbiota-targeted therapies against NAFLD in clinical and experimental studies, and critically evaluate novel targets and strategies for treating NAFLD.

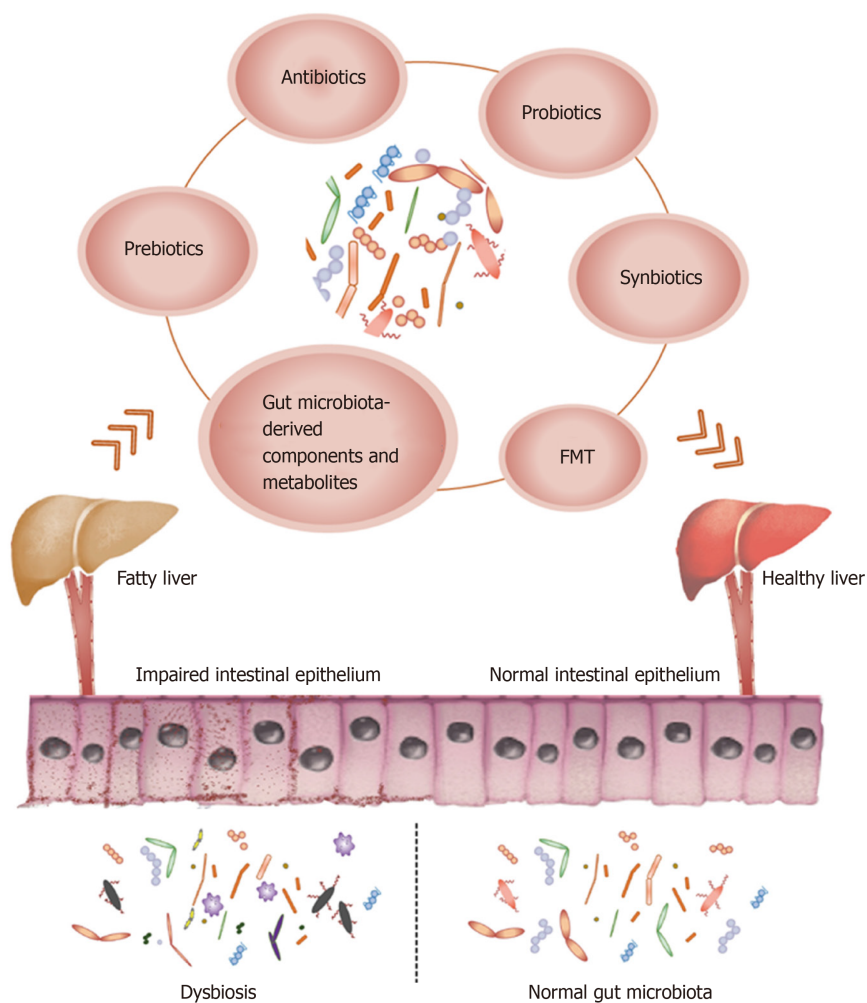


Figure 1 Microbiota-centered therapies against non-alcoholic fatty liver disease. Gut microbiota dysbiosis and impaired intestinal barrier have been elucidated as pathogenic factors in non-alcoholic fatty liver disease. Antibiotics, probiotics, prebiotics, synbiotics, fecal microbiota transplantation, and gut microbiota-derived components and metabolites are important treatments targeting the gut microbiota. FMT: Fecal microbiota transplantation.

ANTIBIOTICS

Antibiotics can eliminate harmful microbiota, and their efficacy has been confirmed in various liver diseases^[21-27]. Since the 1950s^[28,29], neomycin, metronidazole, rifaximin, and polymyxin B have been used extensively for treating cirrhosis and hepatic encephalopathy. In addition, concurrent polymyxin B and neomycin use prevented lipid accumulation in the liver by altering the gut microbiota^[30]. Gangarapu *et al.*^[31] found that short-term administration of antibiotics improved the clinical symptoms in NAFLD/NASH patients by lowering circulating endotoxins as well as serum transaminases. Consistent with this, another study^[32] reported a significant reduction in the levels of transaminase and NAFLD-liver fat score after rifaximin treatment. However, in a recent clinical trial conducted by Ponziani *et al.*^[33], rifaximin showed little therapeutic effects against NASH. This discrepancy could be the result of low drug dose, short duration of the treatment, and small sample size. Antibiotic-induced changes in the gut microbiota can provide valuable insights into its therapeutic utility in various diseases. Specific antibiotics can positively affect the gut microbiota by promoting the growth of beneficial gut bacteria like *Bifidobacteria* and *Lactobacilli*. While short-term antibiotic treatment may have a therapeutic effect, long-term application can lead to the emergence of bacterial resistance, thereby limiting the efficacy of the drug and increasing the risk of secondary infections. Therefore, chronic antibiotic use is not encouraged since they can affect the beneficial gut bacteria and cause intestinal dysbiosis^[34].

PROBIOTICS

Probiotics are non-pathogenic microbes that alleviate gut disorders by restoring the normal microbiota, and provide overall health benefits to the host^[35]. These beneficial bacteria can reduce lipid deposition, endotoxemia, oxidative stress, and inflammation by regulating the expression levels of TNF- α , NF- κ B, and collagen^[35]. Probiotic strains for therapeutic applications are selected on the basis of safety, functionality, and technical requirements^[36]. For instance, some *Streptococcus*, *Lactobacillus*, and *Bifidobacteria* strains can regulate the mucosa-motivated immune system and gastrointestinal inflammation, and promote the growth and survival of gut epithelial cells^[37].

Probiotics have shown significant therapeutic effects on the murine fatty liver model as well. Administering probiotics to mice fed an HFD significantly slowed the progression of hepatic steatosis and fibrosis^[35]. However, most studies on NAFLD rodent models have been aimed at preventing, rather than treating, diet-induced liver disease^[38]. Clinical trials on NAFLD patients have shown that *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* strains play an ameliorative role by restoring the levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT)^[35]. For instance, the intra-hepatic levels of AST and ALT increased significantly in NAFLD patients following 3 mo of treatment with *Lactobacillus bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*)^[39]. MIYAIRI 588, a probiotic *Clostridium butyricum* strain originally from Japan and used widely in Asia, prevented fatty degeneration from progressing to liver cancer in a rat NAFLD model^[40,41]. In addition, co-administration of several probiotic strains, such as the VSL3 formulation including eight probiotic bacterial strains [*S. thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum* (*B. longum*), *Bifidobacterium infantis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *L. bulgaricus*] resulted in greater therapeutic effects compared to any single strain^[42-44]. A randomized controlled trial conducted on overweight children with NAFLD showed significant improvement in the fatty liver condition and BMI following treatment with VSL3. Subsequent studies indicated that the increase in total and active GLP-1 as well as decrease in the plasma levels of S-nitrosothiols, malondialdehyde, and 4-hydroxynonenal was the potential mechanisms underlying the therapeutic effects of VSL3^[45,46]. Furthermore, VSL3 can alleviate chronic liver diseases by protecting the intestinal barrier and reducing endotoxemia and oxidative/nitrosative stress^[46,47].

However, probiotics are primarily derived from bacteria, which raises concerns of biosafety. A few probiotics derived from yeast (*e.g.*, *Saccharomyces boulardii*) have shown encouraging effects, especially when combined with traditional bacterial probiotics^[48]. Further research is needed to optimize the efficacy, safety, and sustainability of probiotics for treating NAFLD.

PREBIOTICS

The International Scientific Association for Probiotics and Prebiotics defines prebiotics as substrates that are broken down by host microorganisms into metabolites^[22] that promote the growth of beneficial bacteria^[49]. Prebiotic feeding is an effective adjuvant therapy for liver diseases, which improves the symptoms by restoring gut microbiota^[10,50]. Oligofructose, a mixture of nondigestible fermentable dietary fiber^[51], reduced liver oxidative stress and inflammation by improving intestinal permeability and tight junction integrity. Prebiotics stimulated the growth of *Bifidobacteria* and normalized plasma endotoxin levels, which improved glucose tolerance and subsequently resulted in weight loss in obese individuals^[52]. Lactulose is another prebiotic that promotes the growth of *Bifidobacteria*, *Lactobacillus*, and Gram-positive bacteria and inhibits the endotoxemic Gram-negative bacteria^[53]. HFD-fed obese mice that were administered lactulose for 6 wk showed reduced inflammation and liver damage, which correlated to decreased circulating levels of lipopolysaccharides^[54]. In addition, the fungal prebiotic chitin-glucan can also limit weight gain, glucose intolerance, liver triglyceride accumulation, and fasting hyperglycemia by modulating the gut microbiota^[55].

The beneficial effects of prebiotics on NAFLD can be attributed to reduced *de novo* lipogenesis, weight and fat loss, improved blood glucose control, restored gut microbiota, and lower inflammation^[56]. Clinical trials have also demonstrated therapeutic effects of prebiotics on NAFLD/NASH progression *via* modulation of glucose homeostasis and lipid metabolism^[57]. In conclusion, prebiotics are a highly suitable therapeutic tool against NAFLD.

SYNBIOTICS

Synbiotics are the combination of probiotics and prebiotics. NASH patients treated with *Bifidobacterium* and fructo-oligosaccharides (FOS) for 6 mo showed significantly lower serum ALT and AST levels compared to the placebo group^[58], indicating the potential advantage of using synbiotics against liver diseases. Another study showed that synbiotic supplementation with seven probiotic strains (*Lactobacillus casei*, *L. bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *B. longum*, and *S. thermophilus*) and FOS for 28 wk, along with healthy lifestyle modifications, was more beneficial (in terms of reduced inflammation and BMI) to NAFLD patients compared to lifestyle changes alone^[59]. Malaguarnera *et al.*^[60] reported that co-administering *B. longum* and FOS for 24 wk combined with a healthy lifestyle significantly decreased NASH activity index and hepatic fat accumulation. Likewise, Safavi *et al.*^[61] found that long-term synbiotic treatment significantly decreased serum lipid levels in obese children. A meta-analysis of 15 randomized controlled trials including a total of 782 NAFLD patients showed that synbiotics markedly attenuated liver steatosis, ALT, AST, high-density lipoprotein, low-density lipoprotein, triglyceride and cholesterol levels, TNF- α expression, the degree of liver stiffness, and homeostasis model assessment-insulin resistance^[62].

FECAL MICROBIOTA TRANSPLANTATION

FMT involves transferring functional microbiomes from the feces of healthy individuals to the gastrointestinal tract of patients with intestinal dysbiosis. It was introduced by Chinese medical and herbal practitioners for treating severe diarrhea and food poisoning^[63]. FMT is an effective therapeutic option for recurrent *Clostridium difficile* infection, as well as liver and metabolic diseases associated with intestinal microbiota dysbiosis. The clinical studies conducted so far on microbiota-targeting strategies, including FMT, in NAFLD patients are summarized in Table 1.

Studies show^[64,65] that transplanting the gut microbiota from lean or obese mice induced phenotypes similar to that of the host, with the microbiota of lean donors significantly reducing adiposity in the obese mice. However, Fischer *et al.*^[66] observed no improvement in the BMI of *Clostridium difficile* infection patients within 12 mo of a single FMT, regardless of the donor BMI. In contrast, overweight patients with metabolic syndrome showed a significant improvement in hepatic (119%) and peripheral (176%) insulin sensitivity 6 wk after receiving microbiota from lean healthy controls compared to the autologous microbiota^[67]. Several studies have demonstrated the therapeutic effects of FMT on type 2 diabetes and ulcerative colitis patients^[68-72], which were associated with restored healthy microbiota, normalized blood lipid levels, and improved insulin resistance.

Based on these reports, we can surmise that FMT is a potential therapeutic option for NAFLD and NASH as well. To determine the role of the gut microbiota in NAFLD development, Le Roy *et al.*^[73] transplanted the feces from HFD responder and non-responder mice into germ-free recipients. The mice that received microbiota from the responder group developed steatosis and showed a high abundance of *Barnesiella* and *Roseburia* in the intestine, whereas microbiota from the non-responder mice markedly increased the abundance of *Allobaculum* in the recipients. Furthermore, an 8-wk FMT intervention^[74] significantly restored the disordered gut microbiota in HFD-induced NASH mouse models by increasing the abundance of beneficial bacteria such as *Christensen* and *Lactobacillus*. It also alleviated endotoxemia, liver steatosis, necrosis, and intra-hepatic inflammation compared to the untreated controls. Consistent with this, metabolic syndrome patients transplanted with the gut microbes of healthy individuals showed increased butyrate production and improved insulin sensitivity^[75], which could be attributed to the higher abundance of beneficial bacteria in the lower gut.

Fecal matter can be implanted through nasogastric tubes, nasojejunoscopy tubes, upper gastrointestinal endoscopy (gastroduodenoscopy), colonoscopy, or retention enema, and the outcomes of these methods differ significantly. In addition, the heterogeneity of donor fecal matter also influences the therapeutic effect. FMT is also associated with the risk of unpredictable infections from the transplanted microorganisms under certain circumstances. Finally, the stability of foreign bacteria into the host gut is limited, which can reduce their long-term survival and therapeutic effects^[76]. Therefore, further clinical trials are warranted to confirm the therapeutic benefit of this strategy.

Table 1 Clinical trials (centered on phase 2 or phase 3) that target the gut microbiota involved in non-alcoholic fatty liver disease

NCT number	Condition(s)	Intervention	Phase	Status	Country
NCT01355575	NAFLD	Antibiotics	Phase 4	Terminated	United Kingdom
NCT02329405	NAFLD	Antibiotics	Phase 4	Completed	Finland
NCT01759628	NAFLD	Antibiotics	Phase 2	Completed	Iran
NCT01712711	NAFLD	Antibiotics	Phase 2	Completed	Iran
NCT01654549	NAFLD	Antibiotics	Phase 2	Completed	Iran
NCT01876108	Fatty liver	Antibiotics	Phase 2	Completed	Iran
NCT02510599	NASH	Antibiotics	Phase 2	Completed	United States
NCT00068094	Fatty liver	Probiotics	Phase 1/Phase 2	Terminated	United States
NCT02972567	Metabolic syndrome/NAFLD	Probiotics	Phase 2	Unknown status ¹	Spain
NCT03511365	NAFLD	Probiotics	Phase 1/Phase 2	Terminated	United States
NCT03585413	Obesity/NAFLD	Probiotics	Phase 3	Recruiting	Germany
NCT04175392	Fatty liver disease	Probiotics	Phase 1/Phase 2	Not yet recruiting	United States
NCT02530138	NASH	Synbiotics	Phase 2/Phase 3	Unknown status ¹	Iran
NCT01791959	NASH	Synbiotics	Phase 2/Phase 3	Completed	Iran
NCT02496390	Diabetes mellitus/NAFLD	FMT	Phase 1/Phase 2	Completed	Canada
NCT02530385	Obesity/NAFLD	FMT	Phase 1/Phase 2	Completed	United States
NCT02741518	Obesity/NAFLD	FMT	Phase 1/Phase 2	Active, not recruiting	United States
NCT02970877	Obesity/NAFLD	FMT	Phase 2	Recruiting	Canada
NCT02050607	Metabolic syndrome/NAFLD	FMT	Phase 3	Unknown status ¹	Italy
NCT02862249	Cirrhosis	FMT	Phase 3	Recruiting	United Kingdom
NCT03014505	Cirrhosis	FMT	Phase 1/Phase 2	Unknown status ¹	China

¹Study has passed its completion date and status has not been verified in more than two years. Data from <https://clinicaltrials.gov/>. FMT: Fecal microbiota transplantation; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

GUT MICROBIOTA-DERIVED COMPONENTS AND METABOLITES

Studies^[77-84] show that the interaction between gut microbiota and their hosts is mediated by various metabolites that are secreted, degraded, or modified by the former, such as short-chain and long-chain fatty acids, amino acids, bile acids, vitamins, and polysaccharides. These metabolites form an intricate signaling network that affects host metabolism and prevents the growth of pathogenic bacteria, and therefore can be utilized to restore the gut microbiota and supplement the effects of FMT or probiotics^[85].

Short chain fatty acids (SCFA) supplementation have shown ameliorative effects in cancer^[86,87], metabolic diseases^[88-90], and other diseases. SCFA is produced during the fermentation of dietary fiber in the gut, and can activate G protein coupled receptor and lower histone deacetylase activity. It is also a fuel for gut epithelial cells and regulates multiple metabolic pathways in the intestine^[91]. SCFA administration in metabolic diseases modulates immune homeostasis, gut hormone secretion, inflammatory response, gut barrier, and other functions^[92-95].

Bile acid (BA) is a cholesterol derivative that is synthesized and conjugated in the liver. It plays a central role in digestion by emulsifying dietary fats and promoting the absorption of lipids and vitamins in the small intestine (mainly the ileum), which affects hepatic lipid accumulation and inflammation. Thus, BA is a critical signaling molecule that functionally connects the intestine and liver. The BA receptor (BAR), also known as FXR, is highly expressed in the liver and intestine and regulates the synthesis of bile acids through a feedback mechanism. A recent study identified TGR5 as another major BAR in the liver^[96]. The underlying mechanisms remain to be elucidated in order to determine whether FXR agonistic or antagonistic effects are beneficial to NAFLD. Amino acid catabolites play a regulatory role in NAFLD by influencing intestinal epithelial barrier and have therapeutic effects on liver function^[96]. Indole propionic acid and other indole-like molecules maintain the integrity of intestinal epithelial barrier^[97] and control inflammation, and can directly act on hepatocytes and liver immune cells^[98].

Gut microbiota-derived metabolites can overcome the major disadvantage of colonization resistance associated with probiotics and FMT. However, metabolite

therapy also has several limitations that ought to be considered^[85]. First, the endogenous gut microbiota and the exogenous metabolites may interact unpredictably, which can aggravate the intestinal dysbiosis or even alter the gut microbiota to produce harmful metabolites. Second, the sudden change in the intestinal levels of the supplemented metabolites may also disrupt the feedback loops of the endogenous metabolites. Long-term supplementation of metabolites can even lead to the emergence of host or bacterial resistance, and thus alter the therapeutic target. Third, the low level of some metabolites in the feces may not truly reflect the status in the intestine, and the suboptimal absorption of oral metabolites in the proximal gastrointestinal tract would decrease their effects on the distal small intestine and colon. In addition, the long-term and systemic effects of these metabolites are unknown and need to be elucidated through detailed pharmacokinetics and pharmacodynamics studies. Finally, bacterial metabolites have very complex chemical structures and some are even volatile, which makes laboratory synthesis technically challenging^[85]. Taken together, the clinical application of bacterial metabolites will have to be supported by strong experimental foundations.

CONCLUSION

NAFLD is a common chronic liver disease that can progress to cirrhosis and HCC, and the prevalence of NAFLD/NASH is increasing globally. The advent of 16S high-throughput sequencing has increased the potential for microbiota-targeted NAFLD/NASH treatment. Apart from bacteria, the intestinal microbiome includes fungi, viruses, and archaeabacteria, which are associated with various liver diseases. Therefore, it is logical to target the gut-liver axis, especially the microbiota, in order to alleviate the symptoms of NAFLD. Although conventional antibiotics can modulate NAFLD symptoms, their clinical use is largely limited due to their side effects and the emergence and prevalence of bacterial resistance. Probiotics, prebiotics, and synbiotics are safe and effective alternatives to conventional antibiotics for treating NAFLD. In addition, FMT is also a promising strategy that can reverse the intestinal dysbiosis associated with NAFLD. Novel therapies involving gut microbiota-derived components and metabolites are increasingly being developed for their unique advantages. The next generation microbiota-targeted therapies against NAFLD include genetically engineered microbiota and recombinant metabolites. Furthermore, the genomes of NAFLD patients and possible genetic determinants of therapeutic responses should also be explored to develop more personalized therapies.

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Basic Study

Interleukin-6 compared to the other Th17/Treg related cytokines in inflammatory bowel disease and colorectal cancer

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Abstract

BACKGROUND

The connection between inflammatory bowel disease (IBD) and colorectal cancer (CRC) is well-established, as persistent intestinal inflammation plays a substantial role in both disorders. Cytokines may further influence the inflammation and the carcinogenesis process.

AIM

To compare cytokine patterns of active IBD patients with early and advanced CRC.

METHODS

Choosing a panel of cytokines crucial for Th17/Treg differentiation and behavior, in colon specimens, as mRNA biomarkers, and their serum protein levels.

RESULTS

We found a significant difference between higher gene expression of *FoxP3*, *TGFb1*, *IL-10*, and *IL-23*, and approximately equal level of *IL-6* in CRC patients in comparison with IBD patients. After stratification of CRC patients, we found a significant difference in *FoxP3*, *IL-10*, *IL-23*, and *IL-17A* mRNA in early cases compared to IBD, and *IL-23* alone in advanced CRC. The protein levels of the cytokines were significantly higher in CRC patients compared to IBD patients.

CONCLUSION

Our findings showed that *IL-6* upregulation is essential for both IBD and CRC

conflict of interest regarding the publication of this article.

Data sharing statement: Consent from the patients for data sharing was obtained, and additionally, the presented data are anonymized, and the risk of identification is low. No additional data are available.

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development until the upregulation of other Th17/Treg related genes (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well.

Key words: Inflammatory bowel disease; Colorectal cancer; Cytokines; mRNA; Interleukin-6; Th17/Treg cells

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Core tip: In our paper, we showed that *IL-6* upregulation is essential for both inflammatory bowel disease and colorectal cancer (CRC) development, whereas the upregulation of other Th17/Treg related genes (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for inflammatory bowel disease and prevention of CRC development as well.

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INTRODUCTION

Inflammatory bowel disease (IBD), as a group of chronic relapsing inflammatory conditions of the gastrointestinal tract, is characterized by prolonged activation of the intestinal mucosal immune system, along with the system involvement, which promotes the release of biological markers, such as cytokines^[1]. The initiation and aggravation of the inflammatory process seem to be due to dysregulated immune responses with a parallel increase in the expression of pro-inflammatory cytokines, and deficiency of anti-inflammatory cytokines. The dysregulated homeostasis of pro- and anti-inflammatory signals contributes to persistent intestinal inflammation^[2].

Inflammation plays a substantial role in sustaining and promoting colorectal cancer (CRC) development as well. As Virchow described in 1863, cancer can arise from inflammatory sites, where the risk of CRC development may increase in the conditions of chronic intestinal inflammation, *via* the malignant cell transformation in the surrounding tissue. Furthermore, the inflammatory response shares various molecular mechanisms and signaling pathways with the carcinogenic process, such as apoptosis, increased proliferation rate, and angiogenesis^[3]. Nonetheless, the activation of two major oncogenic transcription factors/pathways, NF-κB and STAT3, drives the process of chronic inflammation and carcinogenesis^[4]. Activation of NF-κB is required for the induction of *IL-6* by many cell types, such as lymphocytes, monocytes, macrophages, myeloid cells, and cancerous cells. Through STAT3 signaling, cell proliferation and survival are assured by inhibiting apoptosis, cell adhesion, angiogenesis, *etc.* However, STAT3 can exert opposite roles in colon carcinogenesis depending on the tumor stage^[4].

Dysregulation of the immune response in patients with IBD and CRC triggers infiltration and accumulation of immune cells that provoke the release of several cytokines, chemokines, and growth factors, which may further influence the inflammation and the carcinogenesis process^[2]. Immune cells, such as T regulatory (Treg) cells, Type 2 macrophages, CD4+ T-helper (Th)-17 cells, CD8+ T cells, NK, *etc.*, can have effects either sustaining inflammation in IBD, as well as promoting or inhibiting CRC cell growth. The same is also valid for the cytokines which provide the cross-talk between immune cells and CRC cells^[5]. Epithelial cells of the colon are both producers and responders to cytokines and chemokines, and those signals can modulate epithelial cell activity by affecting their proliferation, migration, and survival programs^[6]. For example, cytokines, such as IL-6, IL-17, IL-21, TNFα, are believed to contribute to the formation of tumor-supportive microenvironment through mitogenic effects on the epithelial cancer cells^[5]. There are many subtle mechanisms of inflammatory responses and malignancy involving cytokines: An

induction of reactive oxygen species (*i.e.*, TNF α , IL-6, TGF β), inflammation-associated tumor growth through NF- κ B and STAT3 (*i.e.*, TNF α , IL-17, IL-6, IL-23, IL-10), inflammation-associated epithelial-mesenchymal transition (*i.e.*, TGF β , TNF α , IL-6), inflammation-associated angiogenesis (*i.e.*, VEGF, TNF α , IL-6, TGF β), and inflammation-associated metastasis (*i.e.*, TNF α , IL-6, TGF β , IL-10)^[3,7].

This background is the reason why many investigations are concentrated on the molecular patterns and mechanisms for developing IBD and CRC. Since the recent developments for the role of cytokines in the intestinal inflammation are accumulating, there has been an increased interest in the similarities and differences in inflammation and cancer initiation and advances. We were interested in the comparing of cytokine patterns and subtle changes in cytokine milieu in inflamed tissues of IBD patients, as well as in the cancer environment in CRC patients, especially in the clinical context. However, far too little attention has been paid to opportunities for early detection of subtle changes in cytokine milieu in inflamed tissues in IBD and CRC, especially in the clinical context. As the survival of CRC affected patients depends highly on early detection, we were interested in comparing cytokine patterns of IBD patients in active disease with early and advanced CRC. To address this aim, we choose a panel of pro- and anti-inflammatory cytokines with a substantial role in Th17/Treg differentiation and behavior, like IL-6, TGF β , IL-17A, IL-23, IL-10, in colon specimens of IBD and CRC patients, as mRNA biomarkers, and their protein levels in the peripheral blood.

MATERIALS AND METHODS

Patients

By employing standard clinical, laboratory, endoscopic, histopathological, and radiological criteria, two main study groups were distinguished-IBD patients and CRC patients.

Inflammatory bowel disease patients

A total of 18 IBD patients [13 with ulcerative colitis, ulcerative colitis (UC), and 5-with Crohn's disease (CD)] in an active state of disease without immunosuppressive therapy, attending the University Hospital St. Ivan Rilski, Sofia, during 2011-2013, were recruited for the study. The diagnosis of patients was made according to the standard criteria of ECCO Consensus for CD (2010) and UC (2012) based on a set of anamnestic, clinical, laboratory, and instrumental studies. Criteria for the exclusion of the patients from the study were the following but not limited to: any history of colorectal cancer or hereditary cancer, or the presence of any dysplastic or lesions suggestive of tumor tissues during endoscopic and histological examination; any positive result for autoimmune disease markers (*i.e.*, anti-nuclear antibodies), proved infectious diarrhea, any severe systemic or psychiatric illness. The mean age of the IBD patients was 38 ± 14 years, eleven (61%) were women, and seven (39%) were men.

Colorectal patients

A total of 80 patients with CRC, obtained in the University Hospital and Trakia Hospital, Stara Zagora, during 2011-2017, were included in the study. They underwent surgical resection of the tumor with curative intent. The patients had no history of prior surgery for rectal/colon tumors, and no known hereditary cancer, UC, or CD, they did not receive chemotherapy or radiation therapy prior to surgery. The diagnose of CRC was confirmed by the histopathological examination, and TNM classification was performed for tumor grading and staging. According to the TNM classification, CRC patients were stratified as the following: 40 patients with early CRC (8 with 1st stage + 32 in 2nd stage) and 40 patients with advanced CRC (19 with 3rd + 21 with 4th stages). The mean age of the CRC patients was 64.05 ± 9.6 years. The total group of CRC was composed of 49 males (61%) and 31 females (39%). Paired tumoral tissue samples and adjacent non-tumoral mucosa were obtained from 12 patients with early CRC (7 with 1st stage + 5 with 2nd stage) and 18 patients with advanced CRC (10 with 3rd + 8 with 4th stages). The mean age of the patients with early CRC was similar to that of patients with advanced CRC (67.2 ± 7.2 vs 71.1 ± 10.6 ; $P = 0.29$ *t*-test). Participants included 19 men (63%) and 11 women (37%).

Ethical considerations

All patients gave written consent for the study approved by the Ethical Committee of the Medical University of Sofia and University Hospital St. Ivan Rilski. All patients were informed about the purpose of the study.

Specimen collection and preparation

A total of six intestinal samples were collected from each patient with IBD—from paired inflamed (three) and nearby macroscopically non-inflamed areas (three samples). The tissue samples were taken immediately after a routine endoscopic procedure and were stored at -80 °C until processing. Paired tumoral tissue samples and adjacent non-tumoral mucosa of CRC patients were also collected during the surgical procedure and stored at -80 °C until processing.

Three ml of peripheral venous blood from totally 80 CRC patients (40 cases of early CRC and 40 cases of advanced CRC) and 11 IBD patients in the active state of disease without immunosuppressive therapy were collected in sterile tubes, and serum samples were obtained and frozen at -80 °C before use.

RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Total RNA was isolated from tissue samples using a column-based RNA isolation kit (GeneJET RNA purification kit, Thermo scientific). The total amount of RNA was quantified by spectrophotometric analysis (GeneQuant 1300 spectrophotometer, GE Healthcare Life Sciences, Switzerland).

Reverse transcription to cDNA was accomplished manually according to the manufacturer's instructions with the First-strand cDNA Synthesis kit (Thermo Scientific) and High Capacity cDNA Archive kit (Applied Biosystems, United States).

The qRT-PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, United States) using a TaqMan Universal PCR Mastermix, with cDNA, specific PCR primers sets for our target genes, and 6FAM-labeled TaqMan MGB probes. The ID of Taqman gene expression assays were the following: FoxP3 (Hs00203958_m1), IL-10 (Hs00174086_m1), IL-23 (Hs00372324_m1) from Thermo Scientific; IL-17A (NM_002190), IL-6 (NM_000600) and TGFb (NM_000660) from Primerdesign, United Kingdom. Eukaryotic 18S ribosomal RNA (Hs99999903_m1), from Thermo Scientific, was used as an endogenous control.

Measurement of cytokines

Enzyme immunoassays were performed to measure the protein levels of IL-17A, IL-6, IL-23, TGFb1, and IL-10 in patients' sera (Human ELISA kit, Diaclone, Gene probe, France or Quantikine ELISA Kits, R and D systems, Minneapolis, MN, United States).

Statistical analysis

Triplicated PCR samples were analyzed by Sequence Detection System software v.1.3.1., and the results for mRNA expression were obtained by performing the comparative threshold cycle $\Delta\Delta C_t$ method, after normalization to the endogenous control (as a ratio target mRNA/18S ribosomal RNA). Relative quantification analysis (RQ) represents n-fold mean difference relative to a calibrator (adjacent normal intestinal tissue). Results for local gene expression analyses were expressed as means \pm SD. Cytokine levels were presented as a median and interquartile range (25th percentile and 75th percentile). Statistical differences between groups were analyzed using *t*-test or Mann-Whitney *U*-test. A value of $P < 0.05$ was considered significant. Statistical analysis was performed by using StatSoft software v.6. Dr. Tsvetelina Velikova from the University Hospital Lozenetz reviewed the statistical methods of this study.

RESULTS

Local gene expression of pro-and anti-inflammatory genes in IBD and total CRC patients

Table 1 shows the results of gene expression presented as $dC_t \pm SD$ and RQ values of investigated genes in inflamed tissues of IBD patients and tumoral tissue as well. From all studied genes *IL-6* and *IL-17A* were with an approximately equal level of upregulation in both disease-IBD and CRC, although *IL-6* showed a tendency for the increased concentration in IBD and decreased *IL-17A* in comparison to CRC. The upregulation of *IL-23* gene expression was a hallmark only for CRC, where the level of mRNA synthesis was approximately 25 times enhanced than in IBD. The higher expression was also detected for *TGFb1*, *IL-10*, and *FoxP3* in colorectal tissue compared to IBD local expression.

Figure 1 presents a relative quantity of mRNA levels of the investigated genes. It is visible that the highest RQ difference between IBD and CRC patients was for the *IL-23* gene, as well as for the *FoxP3* and *IL-10* genes and the lowest – for the *TGFb1*, while for the gene expression of *IL-6* and *IL-17A* the differences were not significant.

Local gene expression of pro-and anti-inflammatory genes in IBD and early and

Table 1 Local gene expression of pro and anti-inflammatory cytokines in paired inflamed vs non-inflamed tissues of inflammatory bowel disease patients and paired tumoral vs adjacent non-tumoral mucosa of colorectal cancer patients

Genes	IBD			CRC			P value IBD vs CRC
	dCt ± SD		RQ (min-max)	dCt ± SD			
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue	RQ (min-max)	
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	18.62 ± 5.5	21.30 ± 4.3	6.42 (0.47-97.2)	0.025
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	17.27 ± 2.9	18.63 ± 1.9	2.37 (0.211-93.7)	0.007
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.43 ± 5.5	19.17 ± 4.4	6.66 (0.43-202.3)	0.023
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	19.11 ± 3.8	21.07 ± 2.5	3.88 (0.03-512.4)	0.784
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	12.56 ± 2.1	17.55 ± 2.5	28.5 (2.04-219.5)	0.0003
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	24.7 ± 4.3	26.9 ± 3.0	4.69 (0.034-494.9)	0.574

The results are presented as dCt ± SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

advanced CRC patients

Turning now to the comparison of the local gene expression of target genes in early (1st and 2nd stages) and advanced (3rd and 4th stages) cases of CRC to that in active IBD patients, the results are shown in Figure 2A-F. The figure illustrates that the gene expression of all genes was higher in CRC cases (nevertheless early or advanced) except *IL-6* and *IL-17A* for advanced CRC cases.

Comparing the two stages (early and advanced) of CRC patients, the t-test revealed significant elevation of *Foxp3*, *IL-10*, *IL-23A*, and *IL-17A* mRNAs in early cases of CRC compared to the IBD cases (Table 2). The *TGFB1* mRNA was increased in early CRC compared to the IBD cases with borderline significance ($P = 0.057$). *IL-6* was upregulated in both groups of patients in approximately equal amounts (Table 2). When we compared the gene expression of IBD patients and advanced cases of CRC, we saw that the gene expression of *Foxp3*, *TGFB1*, *IL-10*, and *IL-23* was upregulated, although the statistical significance was reached only for *IL-23*, whereas the mRNA expression of *IL-6* and *IL-17* were downregulated (Table 3). Moreover, the mRNA expression of *Foxp3* (RQ = 10.74), *IL-10* (RQ = 8.44), *IL-23A* (RQ = 61.93), and *IL-17A* (RQ = 26.83) in early cases of CRC and *IL-23A* alone in advanced CRC (RQ = 13.96) were overexpressed.

Serum level of pro- and anti-inflammatory cytokines in IBD and CRC patients

The results from the analysis of serum cytokine levels in IBD and CRC patients are compared in Table 4. All cytokines were at a significantly higher level in CRC patients compared to IBD patients. The levels of *TGFB1* were doubled in CRC compared to IBD patients, and about 20 times higher for *IL-10* and almost 40 times higher for *IL-23*.

Further, we have analyzed the cytokine serum levels in IBD patients in the active state of disease without immunosuppressive therapy ($n = 11$) compared to early CRC ($n = 40$) and advanced CRC ($n = 40$) (Figure 3). Non-parametric statistical analysis revealed that all cytokine levels were elevated significantly in early and advanced CRC patients compared to IBD patients, except *IL-17A*, which was reduced in advanced CRC to the similar to IBD levels. Moreover, the levels of *IL-6*, *IL-10*, and *IL-23* were gradually elevated from IBD – to early – advanced CRC (Table 5).

Our previous data on serum levels of the investigated cytokines in healthy individuals were the following: For *TGFB1*: 10028.82 ± 2250.15 pg/mL, for *IL-6*: 0.03 ± 0.02 , for *IL-17*: 0.16 ± 0.14 pg/mL, for *IL-10*: 0.68 ± 0.05 pg/mL, and for *IL-23*: 0.33 ± 0.03 pg/mL^[8].

DISCUSSION

Since the common feature between IBD and CRC-chronic inflammation was established, we aimed to compare some aspects of the molecular signature of immune cells-inflammatory in IBD and tumor-infiltrated in CRC, like the gene expression pattern for cytokines related to the Th17/Treg differentiation. Our findings showed that several cytokines were significantly upregulated in CRC patients when compared

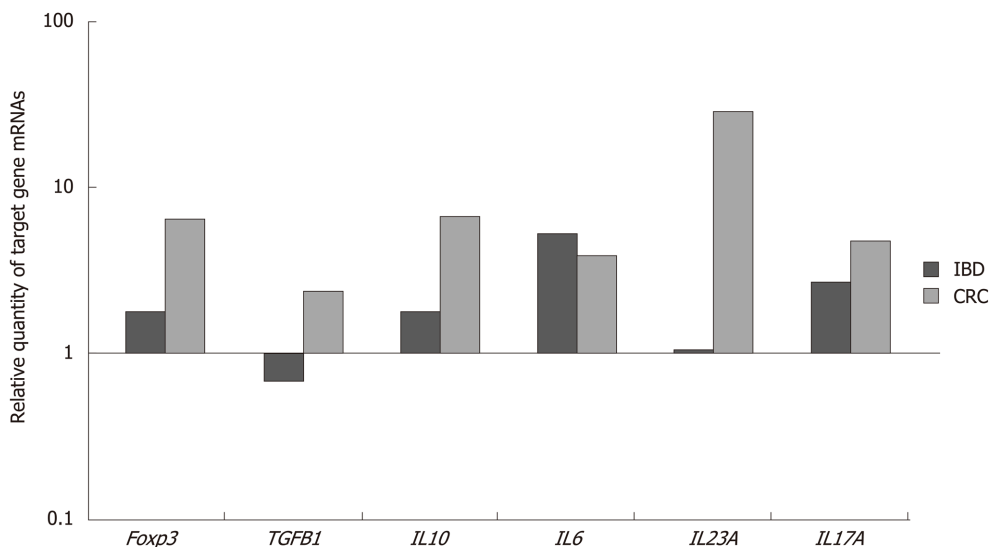


Figure 1 The relative quantity of mRNA levels in inflamed and tumoral tissue calibrated to their adjacent regular counterparts after normalization to endogenous control-18S rRNA. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

to IBD ones. In our study, the cytokines included *TGFB1*, *IL-10*, and *IL-23* in the local microenvironment (tumor tissue vs. inflamed tissue), and *TGFB1*, *IL-10*, *IL-17A*, *IL-6* and *IL-23* in the systemic circulation of patients. The unequal distribution of the cytokines mentioned above supports the general hypothesis that local changes do not mirror the systemic level of inflammation. Nevertheless, a chronic inflammatory response may create an environment which is not only permissive towards cancer development, but that indeed sustains tumor promotion and progression by means of cytokines and growth factor release^[9].

Along with the crucial cytokines, we investigated the mRNA expression of transcription factor *FoxP3*. We found that the expression of *FoxP3* was significantly elevated in the whole CRC group (RQ = 6.42), as well as in the early CRC cases (RQ = 10.74), but not significantly in the advanced stages of CRC (RQ = 4.55). The role of Tregs in inflammation-associated CRC remains elusive. However, the suppression of the immune system may actively hamper host immune surveillance against tumors^[5]. Nevertheless, *FoxP3* expression may suppose a pivotal role for tumor growth, invasion, and spread, in addition to maintaining tumor escape from immune surveillance. Under chronic inflammatory conditions, *FoxP3* + Treg are increased in circulation and accumulate in large numbers in lymph nodes and surrounding tumors. Tumor-infiltrating *FoxP3* + cells are seen primarily during regression of the tumors, *i.e.*, after biologic therapy^[10].

On the other hand, Treg cells may help prevent and delay inflammation-mediated tumor growth as Treg cells exert anti-tumor activity in colitis-associated CRC. In our study, *FoxP3* mRNA levels were higher in the inflamed tissue of IBD patients and even higher in tumoral tissue, especially in the early stages of CRC. This elevation suggests that in the early stages of CRC, there is a more abundant accumulation of *FoxP3* + cells locally, similarly to IBD inflamed tissue.

Treg phenotype contributes significantly to CRC progression, as previously published by Miteva *et al.*^[11] colleagues in 2017. The authors claimed that the upregulation of *FoxP3*, *IL-10*, *TGFB1*, and *IL-6* might be a transcriptional hallmark for CRC metastases, and the gene expression of Treg and Th17 related cytokines in the primary tumor and regional lymph nodes might provide suitable microenvironment sufficient for promoting metastasis^[11]. Previous studies have also shown a significant upregulation of *FoxP3* and *IL-23* in tumor CRC tissue and elevated *IL-10* mRNA on the systemic and local levels of CRC patients^[12].

Recently, in human CRC patients, intratumoral *FoxP3* cells were correlated with a favorable outcome. However, *FoxP3* may be expressed in many cell types, including Th17 cells or tumor cells. Furthermore, Treg cells may not only have diminished efficacy during inflammation but may also differentiate directly into *IL-17*-producing cells^[10]. This way, they may fuel carcinogenic events by contributing to hosting pro-inflammatory responses. Miteva *et al.*^[11] also suggested that the observed elevation of mRNA *IL-17A* with *TGFB1*, *IL-10*, and *IL-6* genes in tumor tissue and regional lymph nodes could be associated with the presence of *FoxP3* + Th17 cells.

Individuals with weakened *IL-10* and Treg cells mediated inhibitory mechanism,

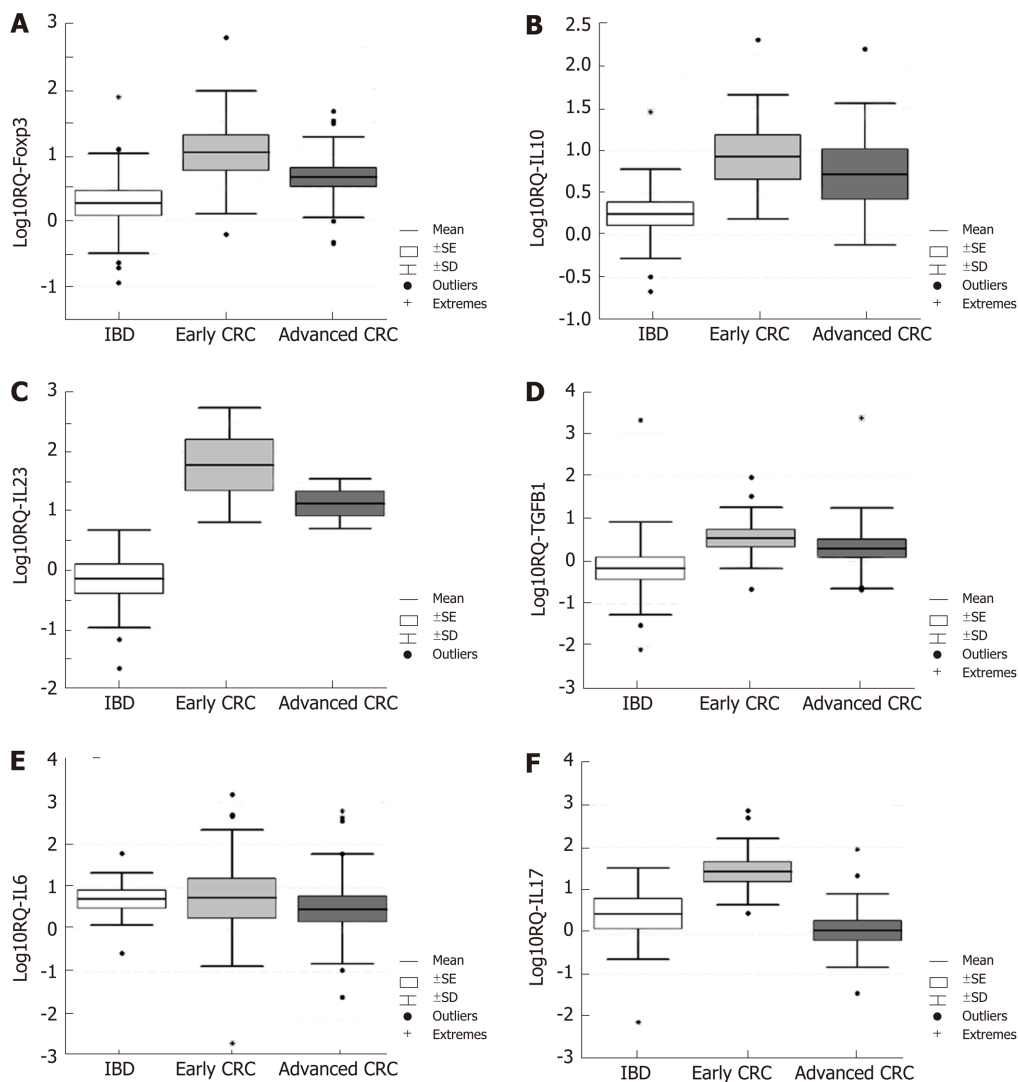


Figure 2 Local gene expression of *Foxp3*, *IL-10*; *IL-23A*; *TGFβ1*; *IL-6* and *IL-17A* in inflammatory bowel disease patients and in early and advanced colorectal cancer cases. A: *Foxp3*; B: *IL-10*; C: *IL-23A*; D: *TGFβ1*; E: *IL-6*; F: *IL-17A*. The relative quantity of target mRNAs in inflamed and tumoral tissue is calibrated to their normal counterparts after normalization to endogenous control-18S rRNA. The results are presented as mean (line) with standard error (box), and standard deviation (whiskers) of log-transformed fold changes (base 10). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

and elevated IL-6 and IL-17, would be more likely to develop uncontrollable inflammation in response to proinflammatory challenges, and thus, more frequently susceptible to inflammation-associated cancers later in life^[10]. In line with these are our results regarding *IL-10* mRNA expression, which we found higher in CRC compared to IBD patients. However, its role remains controversial due to its pro- and anti-tumoral effects. It can be secreted not only by Tregs but also by tumor cells themselves, as well as by tumor-infiltrating macrophages^[13]. *IL-10* inhibits NF-κB signaling; therefore, it can downregulate pro-inflammatory cytokine expression and acts as an anti-tumoral cytokine. *IL-10* can also dampen antigen presentation, cell maturation, and differentiation, allowing tumor cells to evade immune surveillance mechanisms^[14]. *STAT3* can also be activated by *IL-10*, depending on the time frame of *STAT3* activation. *IL-6* leads to a temporary, rapidly declining *STAT3* phosphorylation, whereas *IL-10* induces a sustained one, leading to a pro-tumorigenic effect involving *Bcl-2* upregulation and apoptosis resistance activation^[15].

Some authors reported the average levels of *IL-10* in CRC and CD patients. In our CRC patients, serum levels of *IL-10* were significantly higher than in the IBD patients ($P < 0.0001$) in both early and advanced stages. We suppose that elevated *IL-10* production in tumor tissue is associated with its pro-tumoral activities rather than anti-inflammatory ones. *CD4+* cells insufficient in *IL-10* were preferentially recruited to a Th17 phenotype when faced with a robust pro-inflammatory challenge^[10]. Under conditions of low *IL-10* and elevated *IL-6*, there is weak regulation of inflammation, which may contribute to cancer growth. Individuals with insufficient *IL-10* are more susceptible to the effects of *IL-6* and developing uncontrollable inflammation. In these

Table 2 The local gene expression in inflammatory bowel disease patients compared to that in early colorectal cancer cases

Genes	IBD		Early stages CRC (1 + 2)				P value IBD vs Early CRC
	dCt ± SD		RQ (min-max)	dCt ± SD		RQ (min-max)	
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue		
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	16.98 ± 5.8	20.41 ± 4.5	10.74 (0.615-97.2)	0.023
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	16.99 ± 2.4	18.83 ± 2.3	3.59 (0.222-93.7)	0.057
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.17 ± 5	19.25 ± 4.1	8.44 (1.253-202.3)	0.019
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	18.12 ± 4.7	20.59 ± 1.9	5.55 (0.03-512.4)	0.97
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	11.69 ± 1.9	17.65 ± 2.9	61.93 (2.04-219.5)	0.001
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	22.42 ± 3.6	27.17 ± 2.9	26.83 (2.73-494.9)	0.029

The results are presented as dCt \pm SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

conditions, Treg cells had increased invasion of neoplastic epithelia^[2,10].

It has been reported of upregulation of IL-10 and TGFB1 cytokines in PBMCs of CRC patients preoperative with consecutive downregulation of their expression postoperative suggesting that tumor induces aberrant changes in gene expression, and indicate that the mRNA levels of cytokines were associated with the presence of CRC^[16,17].

The TGFB1 expression under inflammatory conditions may be due to the down-regulation of the body's immune responses in an attempt to control inflammation. As a critical component for both Th17 and Treg differentiation, for us, it was essential to find the expression of this cytokine locally in inflamed (IBD) and tumor (CRC) tissue samples of patients. We observed that TGFB1 was higher in CRC (early and advanced cases) compared to IBD patients. The role of TGFB1 in cancer, however, is complicated and ambiguous, varying by cell type and stages of tumorigenesis. In the early stages of CRC, TGFB1 acts as a tumor suppressor, inhibiting cell cycle progression, and promoting apoptosis. Later, TGFB1 enhances invasion and metastases by inducing epithelial-mesenchymal transition^[18], as well as helps tumor growth by creating an immunotolerant tumor environment^[19]. In our recent study, we showed that the expression of TGFB1 is involved in CRC development and metastasis but depends on gender and genotype^[20]. Thus, we can speculate that increasing the level of TGFB1 is a marker for cancer development or progression.

In our study, the only cytokine, which was approximately equally upregulated in both CRC and IBD, was IL-6. Previously, we found a higher expression of IL-6 in inflamed mucosa of IBD patients^[8] and tumor tissue and regional lymph nodes of CRC patients^[10]. Here, we observed that the differences between mRNA expression of IL-6 locally in IBD and CRC were not significant. We could suggest that this crucial cytokine is of equal importance for both inflammation and tumor development.

IL-6 was shown to promote T cell accumulation in the colon lamina propria by upregulation of anti-apoptotic factors such as BCL-2 and BCL-xL^[21]. IL-6 was also demonstrated to downregulate the tumor suppressor p53 simultaneously, but to upregulate the oncogene c-myc in colon epithelial cells, epithelial-mesenchymal transition, and resistance to cytotoxic stress^[6], and upregulates *Oct4* gene expression by activating IL-6R/JAK/STAT3 signaling pathway^[3]. IL-6 and its related cytokines directly support the growth of colon epithelial cells and repair of intestinal wounds but can also promote the development of CRC^[3]. In summary, IL-6 is a critical tumor promoter during early CRC. Thus, it arises as a potential target in both IBD and CRC.

Some studies showed that CRC patients presented with high levels of IL-6 and VEGF^[22]. Elevated expression of IL-6, which can be detected in patient serum, is linked to increased risk of development of colorectal adenomas^[23] and poor prognosis in CRC^[24,25]. Our CRC patients had elevated serum levels of IL-6 ($P = 0.044$). However, limited studies are existing that might be used to define cut off values for IL-6 as a diagnostic tool.

TGFB1 and IL-6 as crucial cytokines for Th17 differentiation were upregulated in

Table 3 The local gene expression in inflammatory bowel disease patients compared to that in advanced colorectal cancer cases

Genes	IBD			Advanced stages CRC (3 + 4)			P value IBD vs Advanced CRC
	dCt ± SD		RQ (min-max)	dCt ± SD		RQ (min-max)	
	Inflamed tissue	Non-inflamed tissue		Tumoral tissue	Non-tumoral tissue		
<i>Foxp3</i>	14.94 ± 2.65	15.81 ± 2.59	1.82 (0.11-74.85)	19.71 ± 5.2	21.89 ± 4.2	4.55 (0.45-45.1)	0.101
<i>TGFB1</i>	9.52 ± 4.13	8.97 ± 4.4	0.68 (0.03-2.27)	17.46 ± 3.3	18.5 ± 1.6	2.05 (0.211-14.56)	0.176
<i>IL-10</i>	16.87 ± 1.76	17.7 ± 1.5	1.78 (0.22-28.5)	16.69 ± 6.2	19.09 ± 5	5.26 (0.43-158.8)	0.113
<i>IL-6</i>	17.13 ± 3.05	19.53 ± 2.7	5.28 (0.27-61.6)	19.78 ± 3.1	21.39 ± 2.8	3.05 (0.025-434.7)	0.608
<i>IL-23A</i>	18.0 ± 2.09	18.1 ± 2.8	1.07 (0.07-6.61)	13.64 ± 2.1	17.44 ± 2.4	13.96 (3.93-36.28)	0.012
<i>IL-17A</i>	18.9 ± 2.65	20.3 ± 2.9	2.70 (0.07-20.5)	26.62 ± 3.9	26.73 ± 3.2	1.07 (0.034-89.26)	0.350

The results are presented as dCt ± SD and RQ (min-max). IBD: Inflammatory bowel disease; CRC: Colorectal cancer; dCt: Normalized to the endogenous control; RQ: Relative quantity of mRNA levels.

the inflamed tissue of IBD, as well as in tumor tissue of CRC patients. However, the expression of *TGFB1* was higher in CRC patients compared to IBD patients, unlike the *IL-6* expression. Based on these results, we could speculate that the additive upregulation of *TGFB1* expression to the *IL-6* during inflammation could drive to CRC transition. Regarding *IL-17* mRNA expression, as a hallmark of Th17 cells, we found that the cytokine was upregulated in the whole CRC group (RQ = 4.69), especially in early cases in comparison with IBD (RQ = 26.83 *vs* 2.70; *P* = 0.029). Conversely, the RQ level of *IL-17* in advanced CRC cases was lower (RQ = 1.07) than in the IBD group (RQ = 2.70).

It is known that *IL-17A* bridges the adaptive and innate immune system and plays a role in the maintenance of epithelial barrier homeostasis, but in colitis and CRC, its expression is elevated and worsens disease progression^[3]. The role of *IL-17A* as an antitumor or tumor-promoting factor is still incompletely understood; however, increasing evidence support that *IL-17A* is involved in the development of CRC^[26]. *IL-17* family members demonstrated distinct expression patterns in CRC, suggesting a differential role exerted by each member in colon carcinogenesis^[27]. It is tempting to speculate that an inflamed environment dominated by Th17 cells might facilitate cancer development. The involvement of Th17 cells in tumor growth is further sustained by the demonstrated role of *IL-6* in this process^[9].

IL-17A has shown a dual role in controlling neoplastic cell growth – it can inhibit tumor growth in some murine models, while it promotes malignant cells in mouse models of spontaneous intestinal cancer^[28,29]. It can also stimulate tumor growth by its proangiogenic effect via enhancing the production of VEGF, basic fibroblast growth factor, and hematopoietic growth factor, and it has an impact on cancer-infiltrating stem cells^[26]. *IL-17A* contributes to the tumor-initiating stage in the advancement of colitis-associated CRC due to the STAT3 *IL-6* induced Th17 differentiation^[26]. *IL-17* overexpressed in tumors from colitis-associated cancer patients and is associated with angiogenesis and poor prognosis markers. It is secreted in tumors by macrophages/monocytes CD68+; Th17 and Treg FoxP3+*IL-17*+ cells^[30].

The clinical implication of *IL-17A* is investigated by some authors, as they stated that measuring *IL-17A* in serum samples from CRC patients might be a valuable tumor marker^[31], but it is not correlated with the TNM parameters of CRC. We have also found elevated levels of *IL-17A* in the serum of CRC patients compared to IBD patients (*P* = 0.034), which may be useful in the follow up of IBD patients and predicting CRC progression.

The blockade of *IL-17A* leads to a substantial modification in the microenvironment of *IL-17A* related inflammation and tumors^[26,28,29]. And also, targeting *IL-17A* with anti-angiogenic therapeutics may be beneficial for the patients^[26,32].

IL-6 alone can be sufficient to drive Th17 cells to secrete cytokines, whereas *IL-23* is required for providing Th17 cells, a pathogenic phenotype^[6]. In our study, *IL-23* was the only cytokine for which mRNA expression was significantly higher in CRC (both early and advanced cases) than in IBD patients. Despite that different animal models suggest the protective role of *IL-23* in induced CRC in mice, recently published papers have been shown the neoangiogenesis property of *IL-23*, particularly for human CRC development^[16,33,34]. One possible mechanism could be the enhancement of the intestinal inflammation mediated by the Th17 axis maintained from *IL-23*. Moreover, *IL-23* has been found to be overexpressed in a number of different human cancers

Table 4 Serum levels of pro and anti-inflammatory cytokines in inflammatory bowel disease and colorectal cancer patients

Cytokines	IBD patients	CRC patients	P value (U-test)
TGFB1 (ng/mL)	11.19 (9.36-20.99)	22.57 (18.08-29.65)	0.0008
IL-10 (pg/mL)	0.4 (0.0-2.8)	8.29 (6.75-15.12)	0.000001
IL-6 (pg/mL)	0.0 (0.0-2.7)	3.21 (2.03-4.19)	0.012
IL-23A (pg/mL)	0.7 (0.0-3.7)	27.55 (25.35-33.0)	0.000001
IL-17A (pg/mL)	0.0 (0.0-0.26)	2.85 (0.0-9.0)	0.034

The results are presented as median (IQR: 25%-75% percentile). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

compared to normal adjacent tissues, suggesting that IL-23 is essential for tumor-promoting pro-inflammatory processes and as well as the failure of the adaptive immune surveillance to infiltrate tumors^[12,33]. However, IL-12p40 cytokine was evaluated as a useful prognostic marker for survival, unlike IL-23, which had no outcome prognostic value^[16].

Nevertheless, we found that the level of IL-23 was about 40 times higher in CRC patients than IBD patients ($P < 0.001$). Recent data have shown that CRC progression was closely associated with infiltration with Th17 cells, and the central cytokines related IL-6, TGFB1, and IL-23^[35,36]. CRC generates not only the local inflammatory microenvironment, named as tumor-elicited inflammation, but also promotes systemic changes that are favorable for cancer progression. Here, if we compared the serum levels of IL-23 in CRC patients to the average levels of healthy volunteers, the concentrations were 80 times higher in CRC.

Suppression of these cytokines was found to improve the symptoms of IBD to CRC progression^[21,37,38]. This can be accomplished either with anti-cytokine drugs or immunosuppressive agents. Chemoprevention with anti-inflammatory agents and immunomodulatory drugs has been shown to reduce the risk of developing CRC on the grounds of inflammation by lowering the level of produced cytokines^[39,40]. We have also proven the results regarding treatment with immunosuppressive drugs as more beneficial in reducing inflammation in IBD patients via driving cytokine expression to restore immune regulation^[20].

The current study was limited by a relatively small number of IBD patients included. We used a convenient sample of IBD patients with no immunosuppressive therapy to avoid bias. However, caution must be applied, and the hypothesis and findings should be tested with a larger sample size.

Secondly, our study explores two cohorts of patients-IBD and sporadic CRC. Although sporadic colon carcinogenesis and colitis-associated carcinogenesis share similar immune-related mechanisms^[41], we cannot exclude differences in the critical molecular mechanisms underlying these two types of CRC.

In conclusion, with this cytokine panel, we documented significant changes in genes related to Treg/Th17 development when comparing mRNA expression profiles of IBD and CRC (early and advanced) patients. Our findings showed that IL-6 upregulation is essential for both IBD and CRC, whereas the upregulation of genes related to Th17/Treg differentiation and behavior (TGFB1, IL-10, IL-23, and transcription factor FoxP3) is a crucial primarily for CRC development. Altogether we recorded marked differences in the distribution of investigated gene local expression, and the significance of these results could be used mainly in the effort to establish reliable and efficient methods for personalized therapies. Thus, the significantly upregulated IL-6 could be a potential drug target for IBD and prevention of CRC development as well.

Table 5 Serum levels of anti and pro-inflammatory cytokines in inflammatory bowel disease patients compared to early and advanced colorectal cancer cases

Cytokines	IBD	Early CRC	P value (U-test), IBD vs early CRC	Advanced CRC	P value (U-test), IBD vs advanced CRC
TGFB1 (ng/mL)	11.19 (9.36-20.99)	22.74 (18.17-27.98)	0.001344	21.51 (15.5-31.05)	0.0031
IL-10 (pg/mL)	0.4 (0.0-2.8)	7.73 (6.0-12.71)	0.000006	8.84 (7.2-19.81)	0.000002
IL-6 (pg/mL)	0.0 (0.0-2.7)	3.06 (2.03-4.19)	0.0153	4.19 (2.15-8.4)	0.044
IL-23A (pg/mL)	0.7 (0.0-3.7)	25.7 (24.6-32.5)	0.000024	30.5 (26.3-33.5)	0.000009
IL-17A (pg/mL)	0.0 (0.0-0.26)	3.62 (0.0-8.23)	0.0086	1.2 (0.0-12.08)	0.178

The results are presented as median (IQR, 25%-75% percentile). IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

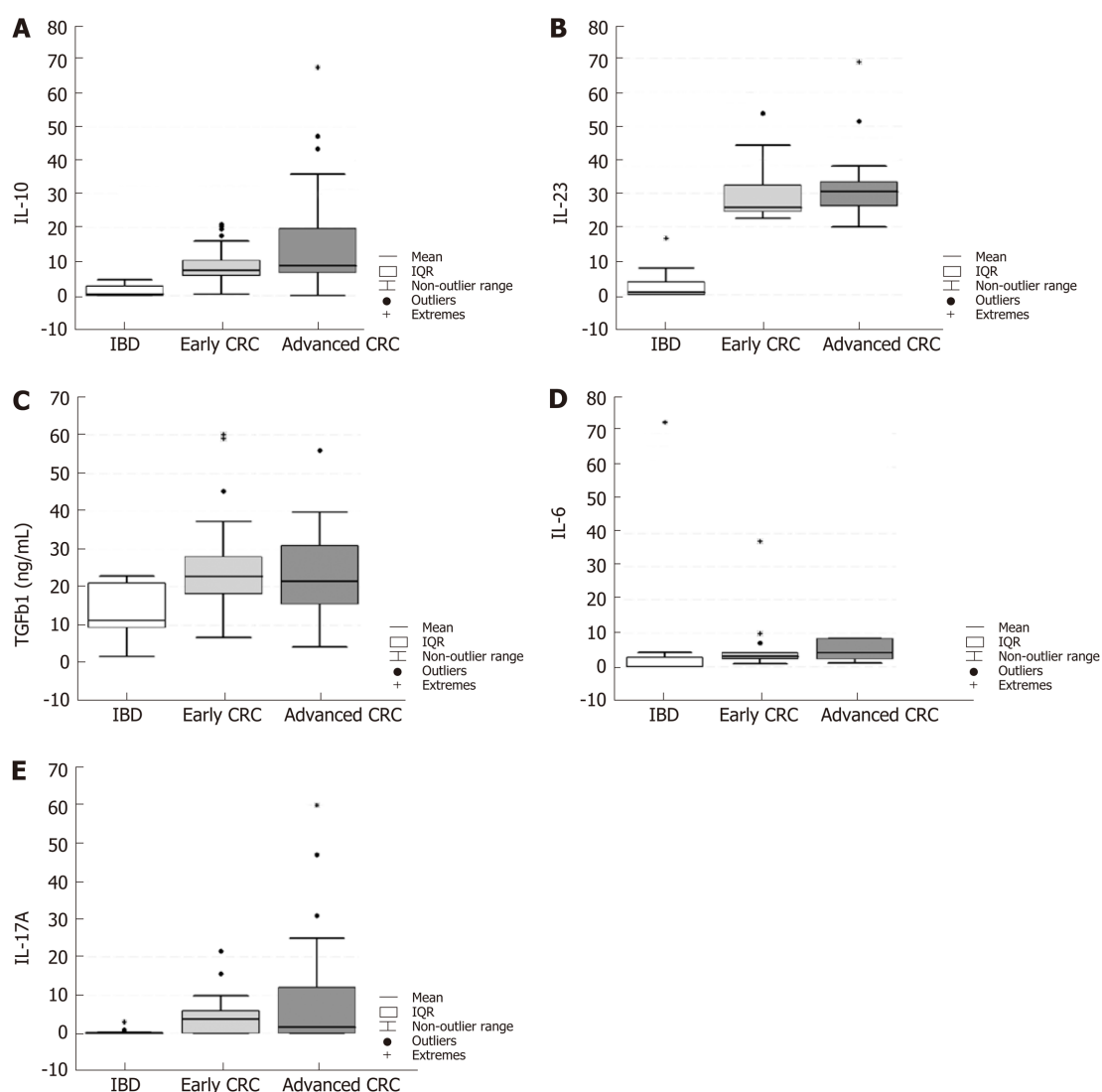


Figure 3 Serum levels of anti- and pro-inflammatory cytokines. A: IL-10; B: IL-23A; C: TGFB1; D: IL-6; E: IL-17A. In inflammatory bowel disease patients compared to early and advanced colorectal cancer cases. The results are presented as median with 25%-75% percentile. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

ARTICLE HIGHLIGHTS

Research background

Since various molecular mechanisms and signaling pathways are common for the carcinogenic process and inflammatory bowel disease, including accumulation of immune cells and the release of several cytokines, chemokines, and growth factors, we were interested in the comparing of cytokine patterns and subtle changes in cytokine milieu in inflamed tissues of inflammatory bowel disease (IBD) patients, as well as in the cancer environment in colorectal cancer (CRC) patients. However, far too little attention has been paid to opportunities for early detection of subtle changes in cytokine milieu in inflamed tissues in IBD and CRC, especially in the clinical context. In line with this, we were searching for mRNA cytokine patterns of IBD patients in active disease compared to early and advanced CRC, to obtain data on in the similarities and differences in inflammation and cancer initiation and advances.

Research motivation

Dysregulation of the immune response in patients with IBD and CRC triggers infiltration and, which may further influence the inflammation and the carcinogenesis process. Since the recent developments for the role of cytokines in the intestinal inflammation are accumulating, there has been an increased interest in their role. Furthermore, the survival of CRC affected patients depends highly on early detection. Thus, establishing some of the parameters that can be detected and followed-up and used as prognostic factors or determining the treatment options can improve the survival rate and quality of life of CRC patients.

Research objectives

Our objectives were: (1) To assess the mRNA cytokine levels in inflamed tissues of IBD patients, as well as in the cancer environment in CRC patients (at early and advanced stages); and (2) To compare serum protein levels of the respective cytokines in both groups of patients. The objectives were chosen to compare cytokine patterns of active IBD patients with early and advanced CRC by selecting a panel of cytokines crucial for Th17/Treg differentiation and behavior (IL-6, TGFb, IL-17A, IL-23, IL-10), as well as the transcription factor FoxP3, in colon specimens, as mRNA biomarkers, and their serum protein levels. Future directions of this study could be the follow-up of IBD patients for developing dysplastic lesions or CRC, where the proposed biomarkers can be evaluated as a prognostic factor.

Research methods

To address the aims of the study, we choose a panel of pro- and anti-inflammatory cytokines with a substantial role in Th17/Treg differentiation and behavior, in colon specimens of IBD and CRC patients, as mRNA biomarkers, and their protein levels in the peripheral blood. We used RNA extraction, reverse transcription, and quantitative polymerase chain reaction, as well as enzyme immunoassays to measure the protein levels of IL-17A, IL-6, IL-23, TGFb1, and IL-10 in patients' sera.

Research results

We found a significant difference between higher gene expression of *FoxP3*, *TGFb1*, *IL-10*, and *IL-23*, and approximately equal level of *IL-6* in CRC patients in comparison with IBD patients. After stratification of CRC patients, we found a significant difference in *FoxP3*, *IL-10*, *IL-23*, and *IL-17A* mRNA in early cases compared to IBD, and *IL-23* alone in advanced CRC. The protein levels of the cytokines were significantly higher in CRC patients compared to IBD patients.

Our findings contribute to the research in the field by finding that *IL-6* upregulation is essential for both IBD and CRC development until the upregulation of other Th17/Treg related genes (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development. The significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well. However, more research are needed regarding the role and targeting of *IL-6* in CRC patients.

Research conclusions

With the chosen cytokine panel, we documented significant changes in genes related to Treg/Th17 development when comparing mRNA expression profiles of IBD and CRC (early and advanced) patients. Our findings showed that *IL-6* upregulation is essential for both IBD and CRC, whereas the upregulation of genes related to Th17/Treg differentiation and behavior (*TGFb1*, *IL-10*, *IL-23*, and transcription factor *FoxP3*) is a crucial primarily for CRC development.

In our study, the only cytokine, which was approximately equally upregulated in both CRC and IBD, was *IL-6*. Previously, we found a higher expression of *IL-6* in inflamed mucosa of IBD patients and tumor tissue and regional lymph nodes of CRC patients. Here, we observed that the differences between mRNA expression of *IL-6* locally in IBD and CRC were not significant. We could suggest that this crucial cytokine is of equal importance for both inflammation and tumor development, *i.e.*, we hypothesized that *IL-6* is a critical tumor promoter during early CRC.

Thus, the significantly upregulated *IL-6* could be a potential drug target for IBD and prevention of CRC development as well, with a significant potential for the clinical practice.

However, since we used a convenient sample of IBD patients with no immunosuppressive therapy to avoid bias, caution must be applied, and the hypothesis and findings should be tested with larger sample size. Secondly, although sporadic colon carcinogenesis and colitis-associated carcinogenesis share similar immune-related mechanisms, we cannot exclude differences in the critical molecular mechanisms underlying these two types of CRC.

Research perspectives

The most important lesson learned from this study is the crucial role of IL-6 for both IBD and CRC. It is well-known that elevated IL-6 is linked to increased risk of development of colorectal adenomas and poor prognosis in CRC, but limited data is available regarding the role of IL-6 in IBD towards CRC. Besides, inadequate studies are existing that might be used to define cut off values for IL-6 as a diagnostic tool. In line with this, future directions should include studies exploring the diagnostic and therapeutic potential of IL-6.

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Basic Study

Mutation analysis of related genes in hamartoma polyp tissue of Peutz-Jeghers syndrome

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Abstract

BACKGROUND

Peutz-Jeghers syndrome (PJS) is a rare disease with clinical manifestations of pigmented spots on the lips, mucous membranes and extremities, scattered gastrointestinal polyps, and susceptibility to tumors. The clinical heterogeneity of PJS is obvious, and the relationship between clinical phenotype and genotype is still unclear.

AIM

To investigate the mutation status of hereditary colorectal tumor-associated genes in hamartoma polyp tissue of PJS patients and discuss its relationship with the clinicopathological data of PJS.

METHODS

Twenty patients with PJS were randomly selected for this study and were treated in the Air Force Medical Center (former Air Force General Hospital) PLA between 2008 and 2017. Their hamartoma polyp tissues were used for APC, AXIN2, BMPR1A, EPCAM, MLH1, MLH3, MSH2, MSH6, MUTYH, PMS1, PMS2, PTEN, SMAD4, and LKB1/STK11 gene sequencing using next-generation sequencing technology. The correlations between the sequencing results and clinical pathological data of PJS were analyzed.

RESULTS

Fourteen types of LKB1/STK11 mutations were detected in 16 cases (80.0%), of which 8 new mutations were found (3 types of frameshift deletion mutations: c.243delG, c.363_364delGA, and c.722delC; 2 types of frameshift insertions: c.144_145insGCAAG, and c.454_455insC; 3 types of splice site mutations: c.464+1G>T, c.464+1G>A, and c.598-1G>A); 9 cases (45.0%) were found to have

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18 types of heterozygous mutations in the remaining 13 genes except *LKB1/STK11*. Of these, *MSH2*: c.792+1G>A, *MSH6*: c.3689C>G, c.4001+13C>CTTAC, *PMS1*: c.46C>t, and c.922G>A were new mutations.

CONCLUSION

The genetic mutations in hamartoma polyp tissue of PJS are complex and diverse. Moreover, other gene mutations in PJS hamartoma polyp tissue were observed, with the exception of *LKB1/STK11* gene, especially the *DNA mismatch repair gene* (*MMR*). Colorectal hamartoma polyps with *LKB1/STK11* mutations were larger in diameter than those with other gene mutations.

Key words: Peutz-Jeghers syndrome; *STK11* gene; *LKB1* gene; Sequencing; Genetic analysis

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Core tip: Peutz-Jeghers syndrome (PJS) is currently considered an autosomal dominant inherited disease caused by germline mutations of the *LKB1/STK11* gene. Gastrointestinal hamartoma polyps are one of the most common clinical manifestations. In order to investigate the mutation status of these familiar genetically-related genes in PJS hamartoma polyp tissues, high-throughput sequencing was used to analyze the mutations of related genes in PJS hamartoma polyps. In addition, the relationships between the mutation status and the clinical pathological data of PJS are discussed.

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INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant inherited disease. The main manifestation of PJS is hamartoma polyps throughout the gastrointestinal tract^[1,2]. It is believed^[3-5] that germline mutations of the tumor suppressor gene *LKB1/STK11* are involved in the etiology of PJS. The encoded product of *LKB1/STK11* gene is a serine/threonine protein kinase which is widely distributed in various tissues^[6,7] and plays an important role in regulating cellular energy metabolism, chromatin remodeling, DNA damage response, cell cycle arrest, p53-mediated apoptosis, as well as cell polarization^[8-10]. Although PJS is a rare clinical disease, these hamartoma polyps can cause serious clinical damage and obvious heterogeneity of clinical phenotypes. Therefore, it is necessary to study the mutations of *LKB1/STK11* gene and other hereditary colorectal tumor-associated genes in PJS hamartoma polyp tissue to investigate the correlation between genotype and phenotype. Twenty patients with PJS were randomly selected for this study, and were treated in the Air Force Medical Center (former Air Force General Hospital) PLA between 2008 and 2017. Fourteen genetically-related genes (*APC*, *AXIN2*, *BMPR1A*, *EPCAM*, *MLH1*, *MLH3*, *MSH2*, *MSH6*, *MUTYH*, *PMS1*, *PMS2*, *PTEN*, *SMAD4*, *LKB1/STK11*) were sequenced in hamartoma polyp tissue from these patients using next-generation sequencing technology to determine the mutation status of these familiar genetically-related genes in PJS hamartoma polyp tissues, and examine the relationship between the mutation status of these genes and the clinical pathological data of PJS.

MATERIALS AND METHODS

Clinical data

Twenty patients with PJS were randomly selected for this study, and were treated in the Air Force Medical Center (former Air Force General Hospital) PLA between 2008 and 2017. All patients met the diagnostic criteria for PJS recommended by the National Comprehensive Cancer Network^[11], and complied with the guidelines of the Declaration of Helsinki. The guardians of children and adult patients were informed

of the purpose of the study, and signed an informed consent form. Their complete clinicopathological data were recorded, and hamartoma polyp tissue samples were obtained and preserved, excluding cancerous polyps (Table 1).

Experimental method

The genomic DNA was extracted from PJS polyp tissue using the QIAamp DNA FFPE Tissue Kit microsample genomic DNA extraction kit, and the experiment was performed according to the kit instructions (QIAamp Tissue DNA FFPE Tissue Kit, QIAGEN, QIAGEN Strasse 1407124 Hilden, Germany).

A normalized cDNA library was built using Ion AmpliSeq Library Kit 2.0 according to the manufacturer's instructions. Two types of Ion AmpliSeq custom panels: IAD72340_182_pool 1 and IAD72340_182_pool 2, were used as multiplex PCR primers, which covered all exons and exon-intron junctions of 14 common hereditary colorectal tumor-associated genes (*APC*, *AXIN2*, *BMPR1A*, *EPCAM*, *MLH1*, *MLH3*, *MSH2*, *MSH6*, *MUTYH*, *PMS1*, *PMS2*, *PTEN*, *SMAD4*, *LKB1/STK11*). After amplification, the paramagnetic particle method (AMPure XP Reagent, Beckman, United States) was used to purify the library. The library was quantitatively detected using fluorescence quantitative PCR (ViiA 7 Dx, Life Technologies Holdings PTE Ltd Block, Singapore city, Singapore). Template preparation (Ion OneTouch2) and template enrichment (Ion OneTouch ES) was then performed using an automated template preparation instrument (Ion OneTouch™ 2 system). High-throughput sequencing was performed using sequencer Ion PGM (Life Technologies).

Quality control sequencing data with a target capture rate > 75%, coverage uniformity > 80%, and average sequencing depth > 150× were used as parameters, and the sequencing results were analyzed using Torrent Suite software (Life Technologies; v5.0.4) and compared using the hg19 Human reference genome. The detected gene mutations were annotated with Ion Reporter software (<https://ionreporter.lifetechnologies.com/ir/secure/home.html>) and ANNOVAR package software (<http://wannovar.wglab.org/>).

Candidate verification sites were screened according to the mutation frequency. The dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), 1000 Genomes Project (<http://ftp.ncbi.nih.gov/>) and the genome Aggregation Database (gnomAD, <http://gnomad.broadinstitute.org/>) were used in the population frequency database. Suspect or clear pathogenic sites included in HGMD (version 2017.03, <http://www.hgmd.cf.ac.uk/ac/index.php>) and database frequency < 0.01, and between 0.01 and 0.05 were retained for verification.

Prime3 online software (<http://bioinfo.ut.ee/primer3/>) was used to design PCR primers for candidate verification sites^[12]. The designed primers were synthesized by Xi'an Qingke Biological Company. The primers were detected and purified after amplification, and were sequenced using the AB 3500xl Dx automatic DNA sequencer (Xi'an Qingke Biological Co., Ltd.). The results verified the preliminary screening of candidate sites.

Protein functional prediction of mutant genes using software Polymorphism Phenotyping v2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/index.shtml>), MutationTaster (<http://www.mutationtaster.org/>), Functional Analysis through Hidden Markov Models (FATHMM, <http://fathmm.biocompute.org.uk/index.html>) and Mendelian Clinically Applicable Pathogenicity (M-CAP, <http://bejerano.stanford.edu/MCAP/>) for primary screening candidate sites verified by first-generation sequencing, and software GERP++ (<http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html>) and PhyloP (<http://compugen.bscb.cornell.edu/phast>) were used to make conservative predictions of amino acid evolution. Protein models were built using SWISS MODEL (<https://www.swissmodel.expasy.org/>) online software.

Statistical analyses

Statistical analysis of the data was performed using the SPSS 24.0 software package. The normal distribution measurement data are expressed as the mean ± SD, and the non-normal distribution data are described as the median (interquartile range Q). The number of statistical data and the composition ratio were compared. The χ^2 test or Fisher's exact probability method was used to compare the composition of the groups. In the quantitative data, the time of occurrence of dark spots, the interval between the appearance of dark spots and abdominal symptoms, the age at initial diagnosis and the maximum diameter of polyps, *etc.* were determined. The inter-group comparison of the state distribution data was performed using the Mann-Whitney *U* test or Kruskal-Wallis *H* test. *P* < 0.05 was considered statistically significant.

Table 1 Clinicopathological data of enrolled patients with Peutz-Jeghers syndrome

Case No.	Onset time of pigment spots (yr)	Gender	Family history or not (detail)	Load of gastric polyps	Maxi-mum diameter of gastric polyps (mm)	Load of duodenal and small intestinal polyps	Maxi-mum diameter of duodenal and small intestinal polyps (mm)	Load of colorectal polyps	Maxi-mum diameter of colorectal polyps (mm)	Number of hospitalization times	Number of operation times	Number of intervention times
1	0	Male	Yes (Father)	0	0	1-10	30	Unknown	Unknown	1	1	3
2	0	Male	No	1-10	10	31-40	80	11-20	70	3	1	4
3	7	Female	Yes (Father)	1-10	6	21-30	25	Unknown	Unknown	1	1	4
4	2	Male	No	11-20	17	1-10	25	1-10	40	1	1	4
5	10	Male	Yes (Son)	1-10	5	51-60	50	41-50	25	5	3	12
6	1	Male	Yes (Mother)	1-10	5	1-10	35	1-10	35	2	1	3
7	1	Male	No	1-10	23	1-10	50	Unknown	Unknown	1	0	2
8	1	Male	Yes (Grandmother and mother)	0	0	Unknown	Unknown	51-60	70	1	4	20
9	7	Male	Yes (Father)	0	0	11-20	60	21-30	12	1	1	9
10	13	Female	No	0	0	1-10	40	1-10	10	3	4	9
11	2	Male	No	1-10	8	1-10	25	1-10	6	2	0	4
12	0	Female	No	1-10	15	41-50	60	11-20	50	2	4	9
13	5	Female	Yes (Father and brother)	21-30	15	41-50	60	21-30	60	4	2	9
14	18	Female	No	21-30	5	11-20	30	1-10	6	2	1	5
15	0.8	Female	Yes (Father)	1-10	6	1-10	6	0	0	2	0	4
16	2	Male	Yes (Father)	21-30	20	31-40	45	51-60	45	2	7	21
17	4	Female	Yes (Son)	Unknown	Unknown	21-30	30	1-10	30	1	1	5
18	0	Male	No	1-10	15	Unknown	Unknown	1-10	20	1	0	2
19	5	Female	No	0	0	11-20	20	Unknown	Unknown	1	2	7
20	4	Female	Yes (Sister)	1-10	50	21-30	50	Unknown	Unknown	1	0	7

RESULTS

Mutations of LKB1/STK11 gene

In this patient group, *LKB1/STK11* gene mutations were detected in 16 of 20 cases, with 14 types of mutations, of which 8 new mutations were detected. According to the prediction of Mutationtaster software, 8 types of protein truncation mutations were found in 10 cases (2 types of nonsense mutations detected in 3 cases, 6 types of frameshift mutations in 7 cases). Among them, the frameshift mutations can cause truncation protein mutations (Table 2). These mutations can change protein function and the prediction of amino acid evolution conservation is shown in Table 3.

Mutation of other 13 genes with the exception of LKB1/STK11

In this patient group, 18 types of gene mutations were detected in 9 of 20 cases, all of which were heterozygous mutations (Table 4). The prediction of protein function change and amino acid evolution conservation caused by the mutations are shown in Table 5 and Table 6. It is less likely that the *PMS2* mutation in patient No. 3 and 4 and

Table 2 Mutation status of *LKB1/STK11* gene

Case No.	Allele	Mutation type	Exon/intron	Amino acid change	Base change	New mutation
1	Heterozygosis	Missense	4	p.L167R	c.500T>G	No
2	Heterozygosis	Nonsense	1	p.K84*	c.250A>T	No
3	Heterozygosis	Frameshift deletion	5	p.A241Vfs*46	c.722delC	Yes
4	Homozygous	Frameshift insertion	3	p.Q152Pfs*11	c.454_455insC	Yes
5	Heterozygosis	Frameshift insertion	1	p.Y49Afs*4	c.144_145insGCAAG	Yes
6	Heterozygosis	Missense	5	p.S240W	c.719C>G	No
7	Heterozygosis	Frameshift deletion	1	p.K82Rfs*14	c.243delG	Yes
8	Heterozygosis	Cleavage site	5-6 ¹	/	c.734+1G>A	Yes
10	Heterozygosis	Cleavage site	3-4 ¹	/	c.464+1G>T	Yes
13	Homozygous	Frameshift deletion	3	p.E145Gfs*10	c.426_448delCGTGCCGGAGAAGCGTTTCCCA G	No
14	Heterozygosis	Nonsense	1	p.K84*	c.250A>T	No
16	Heterozygosis	Frameshift insertion	1	p.Y49Afs*4	c.144_145insGCAAG	No
17	Heterozygosis	Cleavage site	4-5 ¹	/	c.598-1G>A	Yes
18	Heterozygosis	Nonsense	1	p.Y49*	c.147C>G	No
19	Heterozygosis	Frameshift deletion	2	p.K122Afs*40	c.363_364delGA	Yes
20	Homozygous	Cleavage site	3-4 ¹	/	c.464+1G>A	No

¹Mutation is located in the intron.

the *AXIN2* mutation in patient No. 7 were pathogenic based on the results of each software.

Relationship between gene mutation and clinicopathological parameters in patients with PJS

Relationship between mutations and family history: Of the 20 patients in this group, 11 had a family history and 9 had no clear family history. The sequencing results showed the following trend (Figure 1): PJS patients with a family history had a higher *LKB1/STK11* mutation rate than those without a family history (81.1% vs 77.8%, *PLKB1/STK11* = 1.000), and the incidence of *LKB1/STK11* truncation mutations was slightly higher than that in those without a family history (54.5% vs 44.4%, *P*_{truncation mutation} = 1.000). In addition, the incidence of mutations in other genes was slightly lower than that in those without a family history (27.3% vs 66.7%, *P*_{remaining genes} = 0.175). However, due to the small sample size in this group, no statistical difference was observed.

Relationship between mutations and age of dark spots: Of the 20 patients in this group, 11 had black spots aged ≤ 3 years and 9 had black spots aged > 3 years. The former was referred to as the early-onset group and the latter as the late-onset group. The sequencing results showed the following trend (Figure 1): Patients with PJS in the early-onset group had a higher *LKB1/STK11* mutation rate than those in the late-onset group (90.9% vs 66.7%, *PLKB1/STK11* = 0.285), and the incidence of *LKB1/STK11* truncation mutations was slightly higher than those in the late-onset group (54.5% vs 44.4%, *P*_{mutation} = 1.000). In addition, the incidence of mutations in other genes was slightly lower than those in the late-onset group (27.3% vs 66.7%, *P*_{remaining genes} = 0.175). However, due to the small sample size in this group, no statistical difference was observed.

Relationship between mutation and clinical pathological parameters: The group was divided according to the presence or absence of *LKB1/STK11* mutations, presence or absence of *LKB1/STK11* truncation mutations, and other gene mutations. The Mann-Whitney *U* test was used to analyze the differences in polyp distribution, polyp load, and internal or surgical intervention. The results showed that the maximum diameter of colorectal polyps was greater in the presence of *LKB1/STK11* mutations (*U* = 32.000, *P* = 0.048), and the others were not statistically different (Table 7).

Follow-up

All patients of this study were followed-up to January 10, 2020. The final follow-up age was 25.9 ± 15.307 years, and the oldest patient was 47 years. The time span from

Table 3 Prediction of protein function and amino acid evolution conservation of *LKB1/STK11*

Case No.	Polyphen-2		Mutation taster		FATHMM		M-CAP		GERP++		phyloP	
	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction
1	1	Probably damaging	1	Pathogenic	-2.5	Damaging	0.591	Damaging	5.6	Conserved	7.91	Conserved
2	/	/	1	Pathogenic	/	/	/	/	3.9	Conserved	8.998	Conserved
3	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
4	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
5	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
6	0.993	Probably damaging	1	Pathogenic	-2.79	Damaging	0.704	Damaging	5.6	Conserved	7.799	Conserved
7	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
8	/	/	/	/	/	/	/	/	/	/	/	/
10	/	/	/	/	/	/	/	/	/	/	/	/
13	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
14	/	/	1	Pathogenic	/	/	/	/	3.9	Conserved	8.998	Conserved
16	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
17	/	/	/	/	/	/	/	/	/	/	/	/
18	/	/	1	Pathogenic	/	/	/	/	3.9	Conserved	3.875	Conserved
19	/	/	1	Pathogenic	/	/	/	/	/	/	/	/
20	/	/	/	/	/	/	/	/	/	/	/	/

Case No. 8, 10, 17, and 20 are cleavage site mutations.

the patient's first admission was 8.9 ± 8.837 years. Five of these patients were re-admitted to our hospital for a total of 14 colonoscopy examinations and treatments.

DISCUSSION

In this patient group, 80.0% (16/20) of PJS cases were found to have *LKB1/STK11* mutations in hamartoma polyps, consistent with previous reports^[13-15]. In addition, 9 patients (45.0%) also had 18 types of mutations in other genes. The total incidence of mutations in this group of patients was 90.0% (18/20). Among them, *LKB1/STK11* gene: c.243delG, c.363_364delGA, c.722delC, c.144_145insGCAAG, c.454_455insC, c.464+1G>T, c.464+1G>A, c.598-1G>A, *MSH2*: C.792+1G>A, *MSH6*: c.3689C>G, c.4001+13C>CTTAC, *PMS1*: c.46C>T, and c.922G>A are newly discovered mutations, which suggest that the genetic mutations in PJS hamartoma polyp tissue are complex and diverse. In addition, we found that the cases with mutations in the exon 5 of *LKB1/STK11* gene were all in the early-onset group and the cases with splice site mutations in the exon 3 were all in the late-onset group. Those with negative *LKB1/STK11* mutations but carrying other gene mutations were all in the late-onset group. This suggests that different clinical phenotypes of PJS may have a different molecular genetics basis. This is worth further study.

The clinical phenotypic heterogeneity of PJS is obvious. With the continuous improvement in gene detection technology, the relationship between genotype and clinical phenotype has become a focus. However, PJS has scattered populations and relatively few cases as it is also a very rare disease in the clinic. A lot of research has been carried out at home and abroad, but no consensus has been reached on the relationship between genotypes and clinical phenotypes. Although this study did not detect a statistically significant mutation frequency in patients with or without a family history due to the small sample size, we found that the colorectal polyps with *LKB1/STK11* mutations were larger ($U = 32.000$, $P = 0.048$). There was no statistically significant relationship between whether *LKB1/STK11* gene was mutated and whether it was a truncation mutation and the patient's polyp distribution, polyp load, polyp size, and medical or surgical intervention. Some studies have demonstrated that MLPA assay technology can improve the detection rate in *LKB1/STK11* gene mutation screening in PJS patients^[16]. If the MLPA assay is performed in patients with negative mutations, there may be new findings. However, we also found that two patients with *LKB1/STK11* gene exon 5 anterior and posterior splicing site mutations had early-onset of pigment spots, and two patients with cleavage site mutations in exon 3 had late-onset of pigment spots, and patients without *LKB1/STK11* gene mutations but

Table 4 Mutation of other 13 genes except *LKB1/STK11* gene

Case No.	Gene	MMR	Type of mutation	Amino acid change	Base change	New mutation
3	<i>MUTYH</i>	No	Missense	p.Ala373Val	c.1118C>T	No
	<i>MLH1</i>	Yes	Missense	p.Val384Asp	c.1151T>A	No
	<i>PMS2</i>	Yes	Missense	p.Thr511Met	c.1532C>T	No
4	<i>MSH6</i>	Yes	Missense	p.Ala1230Gly	c.3689C>G	Yes
	<i>MLH1</i>	Yes	Missense	p.Val384Asp	c.1151T>A	No
	<i>PMS2</i>	Yes	Missense	p.Thr511Met	c.1532C>T	No
7	<i>MLH3</i>	Yes	Non-synonymous SNV	p.Asp1081His	c.3241G>C	No
	<i>AXIN2</i>	No	Non-synonymous SNV	p.Ser738Phe	c.2213C>T	No
9	<i>MSH6</i>	Yes	Missense	p.Glu1163Val	c.3488A>T	No
	<i>APC</i>	No	Missense	p.Met2221Thr	c.6662T>C	No
10	<i>MSH2</i>	Yes	Missense	p.Ile169Val	c.505A>G	No
	<i>MSH6</i>	Yes	Intron insertion	/	c.4001+13C>CTTAC	Yes
	<i>APC</i>	No	Missense	p.Ala2778Ser	c.8332G>T	No
14	<i>MSH2</i>	Yes	Missense	p.Val89Ala	c.266T>C	No
	<i>MSH2</i>	Yes	Cleavage site	/	c.792+1G>A	Yes
	<i>PMS1</i>	Yes	Nonsense	p.Gln16Ter	c.46C>T	Yes
	<i>PMS1</i>	Yes	Missense	p.Val308Ile	c.922G>A	Yes
15	<i>PTEN</i>	No	Missense in 5' untranslated region (UTR)	p.Gln171Glu	c.511C>G	No
19	<i>MSH2</i>	Yes	Missense	p.Leu390Phe	c.1168C>T	No
20	<i>MLH1</i>	Yes	Missense	p.Arg217Cys	c.649C>T	No

with other gene mutations all had late-onset of pigment spots. Limited by the sample size in this study, there was no statistical difference between the two groups, and we may be able to uncover the molecular genetic mechanism of clinical subtypes if the sample size is increased in further studies.

In addition, the mutation rate of *LKB1/STK11* gene in PJS patients has not reached 100% using various sequencing techniques, which may be related to the limitations of current technology, but it is more likely to suggest that PJS is a heterogeneous genetic disease, or that there are signaling pathways related to its development and progression. Moreover, we also found that there were other gene mutations in the PJS hamartoma polyp tissue, in which the *DNA mismatch repair (MMR)* gene is particularly prominent (accounting for 88.9% of all other gene mutations). According to a variety of software predictions, 81.8% (9/11) of them may be pathogenic and conservative in amino acid evolution. These may be the inherent genetic mechanism of the clinical phenotypic heterogeneity of PJS. The MMR system mainly includes proteins such as hMLH1, hMSH2, hMSH3, hMSH6, hPMS1, and hPMS2, which maintain gene stability mainly by repairing mismatched bases and insertion/deletion loops in DNA synthesis^[17-19]. Among them, MSH2 and MSH6, MSH2 and MSH3 constitute MutS α and MutS β , respectively. The former can recognize single base mismatch and insertion/deletion loops, and the latter can recognize 2-8 base insertion/deletion loops. However, MutL α and MutL β are composed of MLHL with PMS2 and PMSL, and their functions are to localize the mismatch site, cooperate with Exo I, proliferating cell nuclear antigen, and DNA polymerase to remove base mismatches and resynthesize the correct DNA^[20]. Functional alterations in MMR may cause microsatellite instability, which can be found in sporadic and hereditary tumors in various tissues^[21-23], and have clear guiding significance for prognosis and drug efficacy prediction in colorectal cancer patients. In particular, only *MSH6* mutation was detected in the PJS hamartoma polyps without *LKB1/STK11* mutation. This also indicates that there may be other mechanisms besides *LKB1/STK11* involved in the occurrence, development and malignant transformation of PJS hamartoma polyps. Therefore, we consider that destruction of the MMR system may play an important role in the development course of some PJS patients, and with the continuous accumulation of DNA replication errors, it leads to an increased risk of malignant transformation in various tissues and organs. This is worthy of further study.

It was reported that the risk of intussusception in PJS patients was 50% at age 20 years, the incidence of intestinal intussusception was 95%, and 80% of intussusceptions manifested as acute abdomen and 92.5% of cases were treated with surgery^[9]. All patients in the present group did not experience intestinal obstruction,

Table 5 Prediction of protein function changes caused by *MSH6* and other gene mutations

Case No.	Gene	Polyphen-2_HDIV		Mutation Taster		FATHMM		M-CAP	
		Score	prediction	Score	Prediction	Score	Prediction	Score	Prediction
3	<i>MUTYH</i>	0.069	Benign	1	Pathogenic	-2.41	Damaging	0.084	Damaging
3	<i>MLH1</i>	1	Probably_damaging	1	Pathogenic	-2.66	Damaging	/	/
3	<i>PMS2</i>	0.03	Benign	1	Polymorphism	1.06	Tolerable	/	/
4	<i>MSH6</i>	1	Probably_damaging	1	Pathogenic	-2.52	Damaging	0.292	Damaging
4	<i>MLH1</i>	1	Probably_damaging	1	Pathogenic	-2.66	Damaging	/	/
4	<i>PMS2</i>	0.239	Benign	1	Polymorphism	1.06	Tolerable	/	/
7	<i>MLH3</i>	1	Probably_damaging	1	Pathogenic	-2.37	Damaging	0.137	Damaging
7	<i>AXIN2</i>	0.121	Benign	0.997	Polymorphism	-0.25	Tolerable	/	/
9	<i>MSH6</i>	0.67	Probably_damaging	1	Pathogenic	-2.12	Damaging	/	/
9	<i>APC</i>	0.156	Benign	0.737	Pathogenic	-2.47	Damaging	0.046	Damaging
10	<i>MSH2</i>	0	Benign	1	Polymorphism	-2.29	Damaging	0.028	Damaging
10	<i>MSH6</i>	/	/	/	/	/	/	/	/
10	<i>APC</i>	1	Probably_damaging	1	Pathogenic	-1.53	Damaging	0.033	Damaging
14	<i>MSH2</i>	0.042	Benign	1	Pathogenic	-2.47	Damaging	0.075	Damaging
14	<i>MSH2</i>	/	/	/	/	/	/	/	/
14	<i>PMS1</i>	/	/	1	Pathogenic	/	/	/	/
14	<i>PMS1</i>	0.329	Benign	0.996	Pathogenic	-1.34	Tolerable	0.03	Damaging
15	<i>PTEN</i>	0.956	Probably_damaging	0.999	Pathogenic	/	/	/	/
19	<i>MSH2</i>	0.148	Benign	1	Pathogenic	-3.07	Damaging	/	/
20	<i>MLH1</i>	1	Probably_damaging	1	Pathogenic	-1.91	Damaging	0.247	Damaging

intussusception or other gastrointestinal emergencies and malignant changes of polyps during the follow-up period, and did not undergo surgical treatment. We believe that high-frequency enteroscopy and microscopic treatment effectively alleviate the progress of the disease and prolong the patient's survival.

Table 6 Prediction of amino acid evolutionary conservation due to mutations in *MSH6* and other genes

Case No.	Gene	GERP++		phyloP	
		Score	Prediction	Score	Prediction
3	<i>MUTYH</i>	5.67	Conserved	6.955	Conserved
3	<i>MLH1</i>	5.67	Conserved	7.336	Conserved
3	<i>PMS2</i>	-3.23	Nonconserved	-0.25	Nonconserved
4	<i>MSH6</i>	5.5	Conserved	7.481	Conserved
4	<i>MLH1</i>	5.67	Conserved	7.336	Conserved
4	<i>PMS2</i>	-3.23	Nonconserved	-0.25	Nonconserved
7	<i>MLH3</i>	4.6	Conserved	5.502	Conserved
7	<i>AXIN2</i>	2.07	Conserved	2.225	Conserved
9	<i>MSH6</i>	5.23	Conserved	8.923	Conserved
9	<i>APC</i>	6.02	Conserved	3.925	Conserved
10	<i>MSH2</i>	-1.25	Nonconserved	1.857	Nonconserved
10	<i>MSH6</i>	/	/	/	/
10	<i>APC</i>	5.92	Conserved	8.947	Conserved
14	<i>MSH2</i>	3.94	Conserved	3.331	Conserved
14	<i>MSH2</i>	/	/	/	/
14	<i>PMS1</i>	4.99	Conserved	7.805	Conserved
14	<i>PMS1</i>	2.11	Conserved	4.333	Conserved
15	<i>PTEN</i>	/	/	/	/
19	<i>MSH2</i>	4.62	Conserved	1.611	Nonconserved
20	<i>MLH1</i>	5.76	Conserved	2.993	Conserved

Table 7 Relationship between gene mutation and clinical pathological parameters

Mutation	Result	Load of gastric polyps	Maximum diameter of gastric polyps (mm)	Load of duodenal and small intestinal polyps	Maximum diameter of duodenal and small intestinal polyps (mm)	Load of Colorectal polyps	Maximum diameter of colorectal polyps (mm)	Number of hospitalization times	Number of operation times	Number of intervention times
<i>LKB1/STK11</i> mutations	U value	28.000	30.000	35.500	26.000	20.500	32.000	36.000	49.000	28.500
	P value	0.885	1.000	0.442	0.878	0.734	0.048	0.750	0.122	0.750
<i>LKB1/STK11</i> truncating mutation	U value	62.500	69.000	49.500	47.000	23.500	35.500	56.000	40.500	35.500
	P value	0.156	0.053	0.436	0.605	0.613	0.397	0.684	0.481	0.280
Other gene mutations	U value	47.500	42.500	39.000	36.000	22.000	19.500	38.000	46.000	41.500
	P value	0.842	0.842	0.965	0.762	0.607	0.388	0.412	0.824	0.552

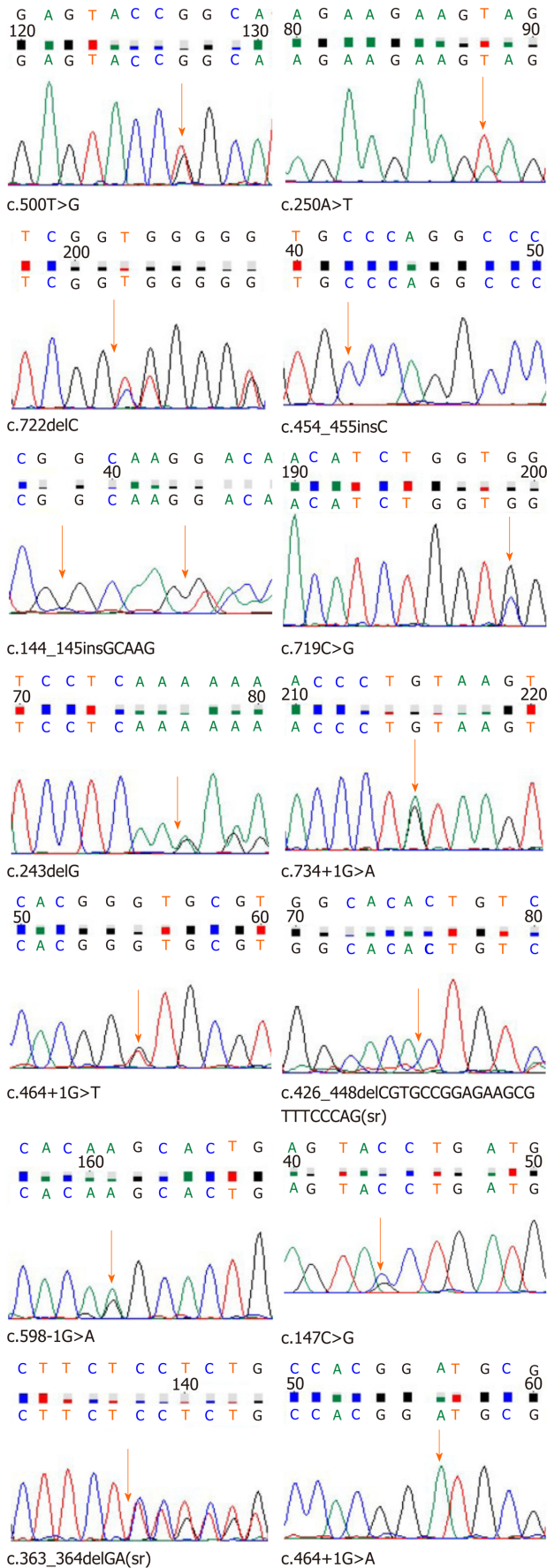


Figure 1 Peak map of *LKB1/STK11* mutation sequencing. The arrow points to the mutation position. "sr" represents reverse sequencing and the remainder is forward sequencing.

ARTICLE HIGHLIGHTS

Research background

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant genetic disease, which belongs to the category of hereditary colorectal cancer. It is currently believed that the occurrence of PJS is closely related to mutations in the *LKB1/STK11* gene, and that different types of mutations have different effects on clinical phenotype. The genetic heterogeneity of PJS is obvious, and no other pathogenic genes have been found except the *STK11* gene, and the relationship between genotype and phenotype is not clear.

Research motivation

This study aimed to investigate the mutation status of hereditary colorectal tumor-associated genes in hamartoma polyp tissue of PJS and discuss its relationship with the clinicopathological data of PJS.

Research objectives

To investigate mutations in genetically-related genes, try to explain the genetic heterogeneity of the disease, and investigate whether the disease has a relatively clear genotype-phenotype relationship.

Research methods

Twenty patients with PJS were randomly selected for this study who were treated in the Air Force Medical Center and their clinicopathological data were collected, including family history, polyp distribution, polyp load, and internal or surgical intervention. Next-generation sequencing technology was used to study the mutation status of the genetically-related genes in PJS hamartoma polyp tissues, and examine the relationship between the mutation status of these genes and the clinical pathological data of PJS.

Research results

LKB1/STK11 gene mutations were detected in 16 of 20 cases, with 14 types of mutations, among which 8 new mutations were detected. 18 types of other gene mutations were detected in 9 of these 20 cases, all of which were heterozygous mutations. There was no statistical difference between mutations and family history, and between mutations and blackspot age. The maximum diameter of colorectal polyps was greater in the presence of *LKB1/STK11* mutations.

Research conclusions

We found a series of gene mutation types in hamartoma polyp tissues of PJS patients, and destruction of the MMR system may play an important role in the development course of some PJS patients. The colorectal hamartoma polyps with *LKB1/STK11* mutations were larger than those with other gene mutations.

Research perspectives

Improvements in gene sequencing technology and the identification of new mutation sites of *STK11* and other possible pathogenic genes are necessary to describe the pathogenesis of PJS at the genetic level. In addition, an investigation into whether the disease has a relatively clear genotype-phenotype relationship is a hot spot for future research.

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Retrospective Study

Iron metabolism imbalance at the time of listing increases overall and infectious mortality after liver transplantation

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Abstract

BACKGROUND

Liver transplantation (LT) is the best treatment for patients with liver cancer or end stage cirrhosis, but it is still associated with a significant mortality. Therefore identifying factors associated with mortality could help improve patient management. The impact of iron metabolism, which could be a relevant therapeutic target, yield discrepant results in this setting. Previous studies suggest that increased serum ferritin is associated with higher mortality. Surprisingly iron deficiency which is a well described risk factor in critically ill patients has not been considered.

AIM

To assess the impact of pre-transplant iron metabolism parameters on post-transplant survival.

METHODS

From 2001 to 2011, 553 patients who underwent LT with iron metabolism parameters available at LT evaluation were included. Data were prospectively recorded at the time of evaluation and at the time of LT regarding donor and recipient. Serum ferritin (SF) and transferrin saturation (TS) were studied as continuous and categorical variable. Cox regression analysis was used to

the routine care of patient does not require signed informed consent for patients included in this kind of study.

Conflict-of-interest statement:

Authors have no conflict of interest to declare.

Data sharing statement:

No additional data are available.

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determine mortality risks factors. Follow-up data were obtained from the local and national database regarding causes of death.

RESULTS

At the end of a 95-mo median follow-up, 196 patients were dead, 38 of them because of infections. In multivariate analysis, overall mortality was significantly associated with TS > 75% [HR: 1.73 (1.14; 2.63)], SF < 100 µg/L [HR: 1.62 (1.12; 2.35)], hepatocellular carcinoma [HR: 1.58 (1.15; 2.26)], estimated glomerular filtration rate (CKD EPI Cystatin C) [HR: 0.99 (0.98; 0.99)], and packed red blood cell transfusion [HR: 1.05 (1.03; 1.08)]. Kaplan Meier curves show that patients with low SF (< 100 µg/L) or high SF (> 400 µg/L) have lower survival rates at 36 mo than patients with normal SF ($P = 0.008$ and $P = 0.016$ respectively). Patients with TS higher than 75% had higher mortality at 12 mo ($91.4\% \pm 1.4\%$ vs $84.6\% \pm 3.1\%$, $P = 0.039$). TS > 75% was significantly associated with infection related death [HR: 3.06 (1.13; 8.23)].

CONCLUSION

Our results show that iron metabolism imbalance (either deficiency or overload) is associated with post-transplant overall and infectious mortality. Impact of iron supplementation or depletion should be assessed in prospective study.

Key words: Iron deficiency; Overload; Cirrhosis; Infection; Death; Ferritin; Transferrin saturation

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Core tip: Iron is an essential element for many biological functions. Its deficiency or overload is associated with poor outcomes in many settings. Few data are available in patients undergoing liver transplantation, and more specifically on infection related deaths. Our study is the first to describe in a large number of patients, the impact of iron metabolism imbalance on mortality after liver transplantation. Our results show that both iron deficiency and overload are significantly associated with increased mortality. Further we show that transferrin saturation higher than 75% is associated with mortality.

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INTRODUCTION

Liver transplantation (LT) is the most effective treatment for patients with end-stage liver disease. However, it is still associated with significant mortality thus promoting the search for associated factors whose optimization could improve survival. Since 2002 in France, liver graft allocation is based on the Model of End-Stage Liver Disease (MELD), which is an objective score improving 3-mo mortality prediction compared to the Child-Turcotte-Pugh score^[1-3]. Other factors of mortality in addition to MELD have been studied to better manage patients waiting for LT like serum sodium^[4-7], renal function^[8], sarcopenia^[9] and ferritin^[10,11].

Serum ferritin (SF) concentration is a reproducible and routinely available biochemical marker. It is the major intracellular iron storage protein and although it correlates with body iron stores, serum level is also influenced by inflammation and cell lysis. Therefore SF increases in patients with elevated body iron stores or in many liver diseases as chronic hepatitis C, alcohol-related liver disease and non-alcoholic steatohepatitis^[12] or in extra-hepatic causes like systemic inflammatory states, systemic immune-mediated diseases (rheumatoid arthritis, adult Still's disease)^[13,14], metabolic syndrome^[15], in case of chronic kidney failure^[16,17], or hematological disease^[18,19].

The relevance of the iron burden in LT was first raised by studies suggesting that patients who undergo LT for cirrhosis related to hereditary hemochromatosis have

higher morbidity and mortality^[20,21], mainly due to an increased risk of infections and recurrence of hepatocellular carcinoma^[22].

Results of studies investigating SF as a risk factor in LT are discordant. Increased SF was associated with an increased risk of death in patients on waiting list and following LT^[10,11].

In contrast, Al-Freah *et al*^[23] using different cut-off values and without considering serum transferrin saturation (TS), showed the lack of influence of SF on wait-list or post-transplant survival, but a significant negative impact of hepatic iron overload on 12 mo' survival. Conversely in a retrospective study addressing iron metabolism and liver iron content, Stuart *et al*^[24] showed that increased hepatic iron concentration was a marker of the severity of the liver disease but was not associated with lower survival after LT.

Further, high SF and TS were found to be risk factors of mortality in other contexts than LT. High SF and TS are associated with increased mortality in acute-on-chronic liver failure^[25] and allogeneic hematopoietic stem cell transplantation^[26]. Similarly in the setting of intensive care, serum iron parameters at admission were correlated with short and long term mortality^[27]. From a biochemical point of view, elevated TS is associated with the appearance of non-transferrin bound iron (NTBI) and labile plasma iron (LPI), which are toxic forms of iron which induce reactive oxygen radicals promoting oxidative stress, leading to cellular damage^[28-30]. Depending on the underlying disease and the assay, NTBI can be detected starting from TS level of 50% to 75%, on the other hand, LPI is always present if TS is higher than 75%^[28,31]. NTBI Imbalance of iron metabolism also fosters siderophilic bacterial infection^[32,33], including pneumonia^[34], with involvement of NTBI^[35].

These discrepant results and the multiple factors influencing or influenced by iron metabolism suggest that more data are required to assess the relevance of iron metabolism imbalance in the management of patients with cirrhosis listed for LT. The aim of our study was to evaluate in a large retrospective cohort of liver transplanted patients the impact of pre-transplant iron metabolism imbalance on post-transplant survival.

MATERIALS AND METHODS

Patients

All patients who underwent LT between January 2001 and December 2011 in our centre and with iron metabolism parameters available at the time of LT listing evaluation were included. Patients with previous history of LT, emergency LT or multiple-organ transplantation were excluded.

Following data were prospectively recorded at the time of evaluation: age, sex, body mass index (BMI) of donor and recipient, cause of cirrhosis, presence of hepatocellular carcinoma, Child-Pugh score, serum sodium, serum iron, TS, serum ferritin, cystatin C, warm and cold ischemia times, perioperative transfusion (red blood cell units, platelets, fresh frozen plasma units), duration of surgery, intensive care unit (ICU) and regular unit length of stay. MELD score was available for all patients after March 2007 and retrospectively calculated for others when INR was available.

Iron metabolism parameters

Iron metabolism parameters (serum iron, SF, transferrin, TS) were routinely measured during pre-transplant evaluation. Serum ferritin was determined using chemiluminescence immunoassay from 2001 to 2009 (ACS 180 Bayer), then turbidimetric assay (Olympus AU 800) from 2009 to the end of the study, internal control and calibration proved consistent measurement and comparability of value between assay.

Transplantation procedure and postoperative care

All patients had orthotopic liver transplantation with inferior vena cava preservation. The graft was harvested from a brain death donor in all cases. No organs from executed prisoners were used. After the procedure, patients were transferred to the ICU until graft function was satisfactory. Routine immunosuppression consisting on low-dose tacrolimus (TAC), mycophenolatemofetil and a short course of corticosteroids was initiated. TAC dosage was initiated at 0.1 mg/kg daily or at 0.06 mg/kg daily in case of concomitant administration of fluconazole and then adapted according to TAC blood through concentration with a target value between 6 and 10 ng/mL. No significant modification regarding the surgical procedure or the postoperative medical care was observed during the study period.

Patient's follow-up

Follow-up data were obtained from the local database and the National Biomedicine Agency database in March 2017. The following complications are prospectively recorded during the follow-up: Anastomotic complications (arterial, venous, biliary), cardiovascular event, renal failure (dialysis), graft rejection.

Patients lost to follow up were considered to be dead. Causes of death were prospectively recorded and categorized as: Cancer (including recurrence of hepatocellular carcinoma), infection related [any causes of bacterial, viral (excluding chronic hepatitis) or fungal infection identified as the main cause leading to death], recurrence of the initial liver disease, graft failure, cardiovascular event, other causes, and undetermined.

This study protocol conformed to the declaration of Helsinki and was approved by the Rennes University Ethics Committee. According to French law, the corresponding database was declared to the "National Committee of Informatics and Freedom" (CNIL, n°96-025).

Statistical analysis

Quantitative variables are described as median [quartiles] and qualitative variables are presented as number and percentage (%).

As SF distribution was skewed and exhibited nonlinearity (according to martingale residuals) in the Cox regression model, it was studied as a categorical variable using tertiles (lower than 100 µg/L - between 100 and 400 µg/L - higher than 400 µg/L). TS was studied as a categorical variable with a cut-off value of 75% as at this level NTBI and LPI are always present^[28].

Clinically relevant variables and variables associated with mortality in univariate analysis (Cox regression) with a *P* value < 0.2 were included in the multivariate analysis. Multivariate Cox regression analysis was performed with backward stepwise selection of variable according to likelihood ratio.

Survival curves were established using the Kaplan-Meier method and compared using the Log-rank test.

Data were analyzed using version 22.0 of SPSS (SPSS Inc., Chicago, IL, United States). *P* < 0.05 was considered significant with a two-tailed test.

RESULTS

Patient characteristics

A total of 553 patients underwent LT during the study period with iron metabolism parameters available at the time of evaluation. Their clinical and biochemical characteristics are described in [Table 1](#).

The median age at evaluation for LT was 55 (49-60.5) years. They were preferentially men (74.9%) with alcohol-related liver disease (61.5%). The median SF was 241 (75.5-593.5) µg/L, 168 patients had SF < 100 µg/L and 200 had SF > 400 µg/L. The median TS was 43.6% (25.1%-74.5%) with 24.6% of patients having a TS higher than 75%. MELD was available in 407 (73.6%) patients and median MELD was 15.1 (10.6-20.7). Median time between listing and LT was 4 (2-9) mo. Median follow-up time after LT was 95 (55-126) mo. At the end of the follow-up period, 5 patients were lost to follow up, and 196 patients died, 38 (6.9%) of died from infection. Of them 13 died of septicemia, 12 of pulmonary bacterial infection, 5 of peritonitis, 3 of angiocholitis, 3 of severe viral infection and 2 of fungal infection. Patient outcomes and causes of death are summarized in [Table 2](#).

Overall mortality

In univariate analysis, variable associated (*P* < 0.2) with overall mortality were: fresh frozen plasma transfusion units (*P* < 0.001), packed red blood cell transfusion (*P* < 0.001), estimated glomerular filtration rate through the CKD Epi Cystatin C equation (*P* = 0.01), duration of surgery (*P* = 0.07), cirrhosis etiology (*P* = 0.07), SF (*P* = 0.08), TS > 75% (*P* = 0.08), ascites (*P* = 0.04), albumin (*P* = 0.10) and Cystatin C (*P* = 0.11).

Results from the multivariate analysis are reported in [Table 3](#). In multivariate analysis, TS higher than 75% was significantly associated with mortality [HR: 1.73 (1.14; 2.63)]. Low SF was significantly associated with mortality [HR: 1.62 (1.12; 2.35)] whereas high SF was not significant.

Because one could consider that abnormal SF or TS may only be markers of advanced liver disease, we performed an analysis entering the MELD score in the final model. MELD was not significantly associated with mortality [HR: 1.00 (0.96; 1.03), *P* = 0.87], this did not change significance of SF or TS, nor significantly changed their Hazard Ratio.

Table 1 Baseline patient characteristics

Variable	n = 553
Age, yr	55 (49-60.5)
Sex male/female	414 (74.9)/139 (25.1)
BMI, kg/m ²	26.2 (23.3-29.7)
Iron metabolism	
Serum iron, µmol/L	21.9 (1.6; 57.9)
Transferrin, g/L	2.10 (1.5-2.5)
Transferrin saturation, %	43.6 (25.1-74.5)
Transferrin saturation > 75%	136 (24.6)
Serum ferritin, µg/L	241 (75.5-593.5)
< 100	168 (30.4)
100-400	185 (33.5)
> 400	200 (36.2)
Cirrhosis etiology	
Alcohol	340 (61.5)
Viral hepatitis C	89 (16.1)
Viral hepatitis B	18 (3.3)
Primary sclerosing cholangitis	20 (3.6)
Primary biliary cholangitis	12 (2.2)
Auto-immune	10 (1.8)
Hemochromatosis	17 (3.0)
Others	47 (8.4)
Hepatocellular carcinoma	214 (38.7)
Child pugh score	
A	206 (39.1)
B	158 (30.0)
C	163 (31.0)
MELD	15.1 (10.6-20.7)
Donor age, yr	49 (37-62)
Donor BMI, kg/m ²	24.3 (21.9-27.6)
Cold ischemia, minutes	592 (446-723)
Perioperative transfusion	
Packed red cell, n	5 (2-8)
Fresh frozen plasma, n	6 (2-9)
ICU length of stay, d	4 (3-7)

For categorical variables, data are given as n (%). For continuous variables, data are given as median (range). BMI: Body mass index; ICU: Intensive care unit; MELD: Model of End-Stage Liver Disease.

Overall survival and serum ferritin

Kaplan Meier survival analysis was performed to explore the relationship between serum ferritin and overall survival. Kaplan Meier survival curves suggest that patients with low SF and high SF have a lower survival than patients with SF within the normal range (Figure 1). This difference was not statistically significant between the three groups over the whole study period ($P = 0.07$). But short-term survival analysis restricted to 6, 12 and 36 mo of follow-up showed a significant difference between the three groups ($P = 0.016$ at 6 mo, $P = 0.026$ at 12 mo and $P = 0.032$ at 36 mo). At 36 mo, patients with ferritin < 100 µg/L and patients with ferritin > 400 µg/L had lower survival than patients with ferritin 100-400 µg/L ($P = 0.008$ and $P = 0.016$ respectively).

Overall survival and TS

Kaplan Meier survival curves show that overall survival was similar between the two groups of TS (lower or higher than 75%) ($P = 0.08$) (Figure 2). However short term survival analysis restricted to 6 and 12 mo of follow-up showed a significant difference with an increased mortality in patients with TS higher than 75% (92.6% ±

Table 2 Patient outcomes

	<i>n</i> (%)
Dead	196
Lost to follow up	5
Causes of death	
Cancer	41 (20.92)
Infection	38 (19.39)
Recurrence of initial liver disease	30 (15.31)
Graft failure	12 (6.12)
Cardio vascular event	28 (14.29)
Others	31 (15.82)
Undetermined	16 (8.16)

1.3 *vs* 86.0% \pm 3.0, $P = 0.012$ at 6 mo and 91.4% \pm 1.4% *vs* 84.6% \pm 3.1%, $P = 0.039$ at 12 mo) suggesting an impact of TS on the early phase after LT.

Factors associated with infection related deaths

Because iron overload and NTBI are thought to promote sepsis, we then addressed the impact of iron metabolism on infection related deaths and infections after LT.

In univariate analysis, factors associated ($P < 0.2$) with infection related deaths were: ICU length of stay ($P < 0.001$), estimated glomerular filtration rate through the CKD Epi Cystatin C equation ($P = 0.03$), recipient BMI ($P = 0.07$), packed red blood cell transfusion ($P = 0.07$), TS $> 75\%$ ($P = 0.15$), SF ($P = 0.16$), bilirubin ($P = 0.17$), and fresh frozen plasma transfusion ($P = 0.17$).

In multivariate analysis using Cox regression (Table 4), increased TS was significantly associated with the risk of infection related deaths [HR: 3.06 (1.13; 8.23)]. SF was associated with infection related deaths with high SF associated with lower infection related death [HR: 0.35 (0.12; 0.98)] whereas low SF was not significant.

DISCUSSION

In our study, we examined the impact of iron metabolism at the time of evaluation for LT on post-transplant mortality. Our results show that TS $> 75\%$ and low SF were risk factors of overall mortality. This suggests that iron metabolism is a double-edged sword as both overload and deficiency are associated with mortality. Kaplan Meier survival curves show that increased mortality in patients with TS $> 75\%$ is seen at 6 and 12 mo after LT. Further our results show that TS $> 75\%$ was significantly associated with infection related deaths after LT.

The first interesting results of our study was the surprising fact that low SF was associated with mortality while high SF was not, contrary to other studies^[10,11]. Because iron metabolism can be either in excess or deficiency situation, we think that both must be considered. Our results show that iron deficiency and overload were both risk factors of overall mortality. This U shape association is consistent with previous finding. Iron deficiency has already been demonstrated as a risk factor of mortality in ICU^[36-38] and as a risk factor of infections because of immune depression induced by iron deficiency^[39-44]. On the other hand, iron supplementation particularly by perfusion is associated with an increased risk of infections^[45-47] and low SF is a protective host mechanism^[48]. This two-way effect could explain the discrepant results regarding the impact of SF on mortality after LT^[11,23].

Weismüller *et al*^[11] showed that SF ≥ 365 $\mu\text{g/L}$ in combination with TS $< 55\%$ before LT was an independent risk factor for mortality following LT, suggesting an impact of both iron overload and other factors (acute phase, immune response). However the cut-off level of $< 55\%$ for TS was chosen by receiver operating curve analysis in a subgroup of patients with SF > 365 $\mu\text{g/L}$ with the goal to distinguish which patients survived. Although this may represent iron deficiency or inflammation, there is little information on the potential mechanisms by which low TS could be associated with increased mortality. Further, multivariate analysis was only performed using a binary variable distinguishing patients with TS $< 55\%$ and SF ≥ 365 $\mu\text{g/L}$ to all others, without describing multivariate results regarding SF or TS separately.

We choose to assess TS from a physiological point of view, in agreement with the appearance and toxicity of NTBI and LPI we choose high TS $> 75\%$ as a cut-off^[28]. This

Table 3 Cox multiple regression analysis for overall mortality

Parameters	HR (95%CI)	P value
Hepatocellular carcinoma (yes/no)	1.58 (1.15; 2.16)	0.004
eGFR CKD EPI Cystatin C (mL/min)	0.99 (0.98; 0.99)	0.01
Cirrhosis aetiology (HCV as reference)		0.028
Alcohol	0.57 (0.39; 0.83)	0.003
Hepatitis B virus	0.50 (0.19; 1.28)	0.14
Other	0.66 (0.41; 1.08)	0.10
Transferrin saturation > 75%	1.73 (1.14; 2.63)	0.01
Ferritin (100-400 µg/L as reference)		0.009
< 100 µg/L	1.62 (1.12; 2.35)	0.01
> 400 µg/L	0.90 (0.59; 1.37)	0.63
Packed red blood cell (per unit)	1.05 (1.03; 1.08)	< 0.001

CI: Confidence interval; HCV: Hepatitis C virus; HR: Hazard ratio; ICU: Intensive care unit.

might be considered a better surrogate of functional iron overload, as it represents a high level of biologically available iron in the bloodstream. Our multivariate analysis taking into account TS > 75% shows that high serum ferritin is not associated with overall mortality. Further high serum ferritin is associated with a lower infection related mortality, while TS > 75% is associated with higher infection related mortality. This emphasizes the paradoxical situation between functional iron overload (TS > 75%) and high serum ferritin that may only reflect inflammation as ferritin is also an acute phase protein.

For Al-Freah *et al*^[23] SF as a categorical variable (> or < 300 µg/L) is not associated with post LT survival, but patients with explant siderosis grade ≥ 2 had inferior 12-mo post-LT survival ($P = 0.03$). As the fast and efficient uptake of NTBI by liver parenchymal cell is well described^[49], and is a major mechanism for liver siderosis, this could be in accordance with our result showing that high TS is a risk factor. However TS was not assessed in this study.

We think that a major limitation of these study is the lack of consideration for iron deficiency. Iron deficiency can affect a significant part of the population, even more in the setting of end-stage liver disease as chronic bleeding and frailty are common. Actually one third of our patient had a serum ferritin lower than 100 µg/L that can be considered as low in this setting. This may be a major cause of discrepancy between studies and this may also explain the non-linearity between SF and mortality that prevent its simple use as a continuous variable in Cox regression analysis.

The use of different SF cut-offs across studies is in part explained by the heterogeneity of biochemical methods used by centres and laboratories and the lack of international standards and reference materials. Moreover, SF may be affected by several factors besides iron metabolism, especially in the context of liver disease and inflammation. Therefore we think one should more consider an indication toward iron deficiency or overload rather than a definite cut-off that is hard to define in this setting.

Iron overload with high SF and TS have been shown to be risk factors of mortality in other situations than LT^[25,27,50,51]. High SF has been demonstrated to be a risk factor of infections after kidney transplantation^[52]. Chow *et al*^[53] recently shown that increased levels of serum iron were independently associated with an increased risk for several types of infection and deaths. Recently, a second study of Chow *et al*^[54] also showed a link between high SF, high Hcpidin and low serum iron, with infection after LT.

Our finding that TS higher than 75% is associated with increased overall and infection related mortality support our initial hypothesis of NTBI and LPI production, a toxic free iron present in case of TS > 75%^[28,30]. Increasing iron availability with high TS and production of NTBI/LPI promotes development of bacterial infection via increased bacterial growth. Further our results suggest that these patients may be more prone to infection in the post-operative period.

Overall our results are in accordance with the fact that iron is an essential nutrient for all organisms but can paradoxically become toxic, inducing oxidative stress in case of iron overload^[29,30]. Iron deficiency leads to immunosuppression, which in turn increases the risk of infections. Conversely, iron overload provokes production of free radicals and oxidative damage and influence pathogen growth.

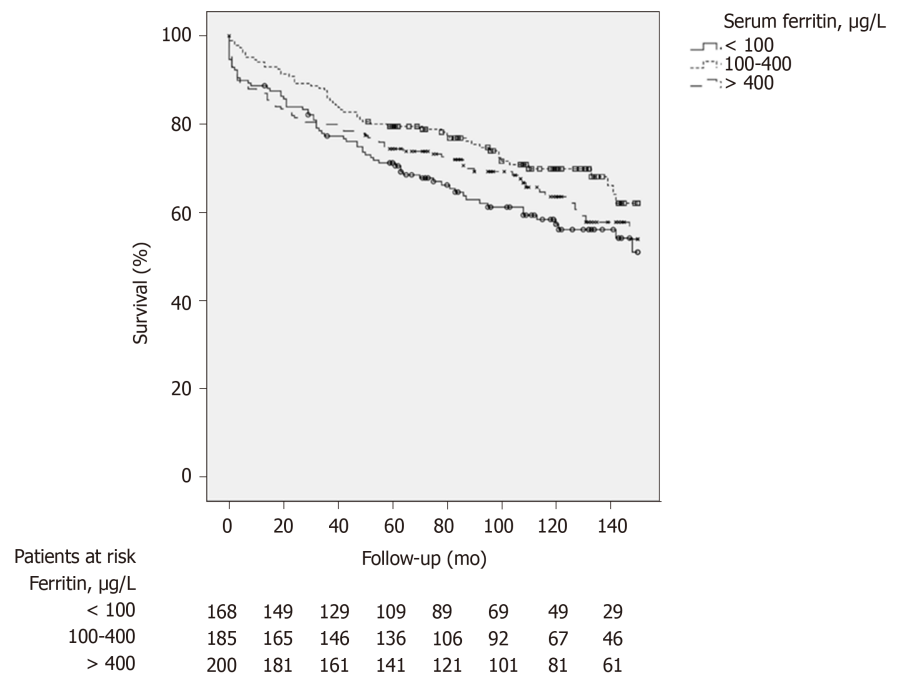


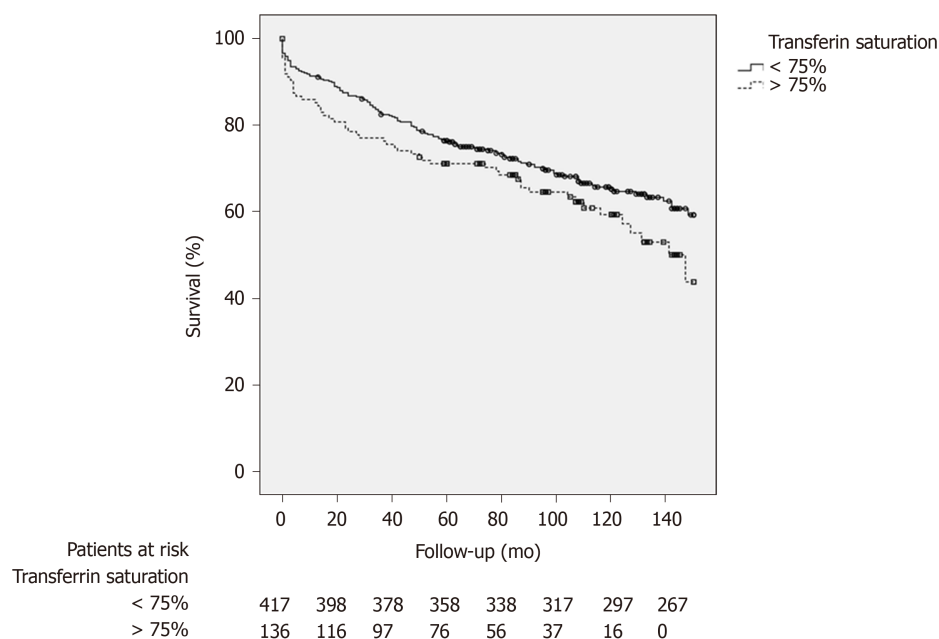
Figure 1 Kaplan Meier survival curves according so serum ferritin.

In conclusion, our results show that imbalance in iron metabolism is significantly associated with sepsis-related and overall mortality in the post-transplant period. The role of iron should be prospectively studied in these patients to identify patients at risk and guide optimization of iron metabolism management to improve post-transplant survival.

Table 4 Multivariate analysis for risk factors of infection associated death

Parameters	HR (95%CI)	P value
Transferrin saturation > 75%	3.06 (1.13; 8.23)	0.02
Ferritin (100-400 µg/L as reference)		0.02
< 100 µg/L	1.74 (0.77; 3.93)	0.17
> 400 µg/L	0.35 (0.12; 0.98)	0.48
eGFR CKD EPI Cystatin C (mL/min)	0.98 (0.97; 0.99)	0.03
ICU length of stay (d)	1.02 (1.01; 1.03)	< 0.01

CI: Confidence interval; HR: Hazard ratio; ICU: Intensive care unit.

**Figure 2** Kaplan Meier survival curves according to serum transferrin saturation.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation (LT) is the best treatment for patients with liver cancer or end stage cirrhosis, but it is still associated with a significant mortality. Therefore identifying factors associated with mortality could help improve patient management. The impact of iron metabolism, which could be a relevant therapeutic target, yield discrepant results in this setting. Previous studies suggest that increased serum ferritin is associated with higher mortality. Surprisingly iron deficiency which is a well described risk factor in critically ill patients has not been considered.

Research motivation

Iron metabolism could be easily corrected before liver transplantation, thus assessing its impact on mortality is critical before designing clinical trials in that purpose.

Research objectives

The main objectives, the objectives that were realized, and the significance of realizing these objectives for future research in this field should be described in detail.

Research methods

Retrospective cohort analysis with Cox multivariate Regression. Survival was also estimated through Kaplan Meier analysis.

Research results

A large number of patients was studied (553) and followed for 95 mo. At the end of follow-up 196 patients were dead, 38 of them because of infections. In multivariate analysis, overall

mortality was significantly associated with transferrin saturation (TS) higher than 75% [HR: 1.73 (1.14; 2.63)], serum ferritin lower than 100 µg/L [HR: 1.62 (1.12; 2.35)], hepatocellular carcinoma [HR: 1.58 (1.15; 2.26)], estimated glomerular filtration rate (CKD EPI Cystatin C) [HR: 0.99 (0.98; 0.99)], and packed red blood cell transfusion [HR: 1.05 (1.03; 1.08)]. Kaplan Meier curves show that patients with low serum ferritin (< 100 µg/L) or high serum ferritin (> 400 µg/L) have lower survival rates at 36 mo than patients with normal SF ($P = 0.008$ and $P = 0.016$ respectively). Patients with TS higher than 75% had higher mortality at 12 mo ($91.4\% \pm 1.4\%$ vs $84.6\% \pm 3.1\%$, $P = 0.039$). Moreover TS higher than 75% was significantly associated with infection related death [HR: 3.06 (1.13; 8.23)].

Research conclusions

Our study is the first to describe the respective impact of iron deficiency in patient undergoing liver transplantation. This suggests that active management of iron deficiency with iron supplementation before liver transplantation could significantly improves outcome after liver transplantation. Further this is the first study showing that high TS is associated with higher short term mortality. This suggests that exposure to toxic forms of iron increase cellular damages in the perioperative period and that treating iron overload before liver transplantation could improves outcome.

Research perspectives

A prospective randomized clinical trial is required to assess the beneficial effect of correction iron metabolism imbalance before liver transplantation.

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Observational Study

Effectiveness of very low-volume preparation for colonoscopy: A prospective, multicenter observational study

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Abstract

BACKGROUND

The effectiveness of colonoscopy strictly depends on adequate bowel cleansing. Recently, a 1 L polyethylene glycol plus ascorbate (PEG-ASC) solution (Plenvu; Norgine, Harefield, United Kingdom) has been introduced on the evidence of three phase-3 randomized controlled trials, but it had never been tested in the real-life.

AIM

To assess the effectiveness and tolerability of the 1 L preparation compared to 4 L and 2 L- PEG solutions in a real-life setting.

METHODS

All patients undergoing a screening or diagnostic colonoscopy after a 4, 2 or 1 L PEG preparation, were consecutively enrolled in 5 Italian centers from September 2018 to February 2019. The primary endpoints of the study were the assessment

statement: The study received Ethics Committee approval and was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of this manuscript.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at marcello.maida@hotmail.it. Participants gave informed consent for data sharing.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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of bowel cleansing success and high-quality cleansing of the right colon. The secondary endpoints were the evaluation of tolerability, adherence and safety of the different bowel preparations. Bowel cleansing was assessed through the Boston Bowel Preparation Scale. Adherence was defined as consumption of at least 75% of each dose, while tolerability was evaluated through a semi-quantitative scale. Safety was systematically monitored through adverse events reporting.

RESULTS

Overall, 1289 met the inclusion criteria and were enrolled in the study. Of these, 490 patients performed a 4 L-PEG preparation (Selgesse®), 566 a 2 L-PEG cleansing (Moviprep® or Clensia®) and 233 a 1 L-PEG preparation (Plenvu®). Bowel cleansing by Boston Bowel Preparation Scale was 6.5 ± 1.5 overall and 6.3 ± 1.5 , 6.2 ± 1.5 , 7.3 ± 1.5 ($P < 0.001$) in the subgroups of 4 L, 2 L and 1 L-PEG preparation, respectively. Cleansing success was achieved in 72.4%, 74.1% and 90.1% ($P < 0.001$), while a high-quality cleansing of the right colon in 15.9%, 12.0% and 41.4% ($P < 0.001$) for 4 L, 2 L and 1 L-PEG preparation groups, respectively. The 1 L preparation was the most tolerated compared to the 2 and 4 L-PEG solutions in the absence of serious adverse events within any of the three groups. Multiple regression models confirmed 1 L PEG-ASC preparation as an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability.

CONCLUSION

This study supports the effectiveness and tolerability of 1 L PEG-ASC, also showing it is an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability.

Key words: Colonoscopy; Bowel preparation; Polyethylene glycol; Polyethylene glycol plus ascorbate; Effectiveness; Tolerability

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Core tip: The effectiveness of colonoscopy strictly depends on adequate bowel cleansing. Nevertheless, bowel preparation is not always well accepted and tolerated by all patients. Recently, a 1 L polyethylene glycol plus ascorbate solution has been introduced on the evidence of three phase-3 randomized controlled trials, but it had never been tested in the real-life. This prospective, multicenter, observational study performed on 1289 patients undergoing screening or diagnostic colonoscopy, confirmed the effectiveness and tolerability of very low-volume preparation for colonoscopy, also showing that it is an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability.

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INTRODUCTION

Colonoscopy is one of the most widely diffused methods for screening of colorectal cancer (CRC), and regular screening is of primary importance since early detection of the cancer is associated with a long-term reduction of disease incidence and mortality^[1,2].

Effectiveness of colonoscopy strictly depends on adequate bowel cleansing, since it can affect the diagnostic accuracy and the adenoma detection rate^[3]. Moreover, recent data show that high-quality bowel cleansing is also necessary to improve the



detection of sessile serrated polyps^[4]. On the contrary, a suboptimal bowel preparation negatively affects the performance of colonoscopy, since it results in the increase of procedural duration, potential greater risks of adverse events (AEs), rescheduling of procedures and higher costs^[4-8]. More recent guidelines recommend the use of high volume or low volume polyethylene glycol (PEG) based regimens as well as that of non-PEG-based agents that have been clinically validated for routine bowel preparation, in a split-dose regimen^[9]. Nevertheless, many solutions require the ingestion of volumes of up to 4 L, which may reduce patients' compliance resulting in a suboptimal bowel cleansing that is still reported in about one-quarter of procedures^[10]. The same issue has been confirmed by a large Italian study showing that inadequate bowel preparation is found in about 17% of colonoscopies^[11], and the results from the United Kingdom screening program show that more than 20% of incomplete procedures are caused by poor preparation^[12].

A very low-volume 1 L PEG plus ascorbate (PEG-ASC) solution (Plenvu; Norgine, Harefield, United Kingdom) has been recently introduced on the market to improve patients' experience in colonoscopy by reducing the total intake of liquids to be consumed.

Development of this very low volume 1 L preparation was made possible by increasing the content of ascorbate, which enhances the laxative effect, permitting the delivery of the solution in a smaller volume.

After the evidence from a first phase-2 study^[13], three parallel phase-3 randomized controlled trials (RCTs) comparing effectiveness of 1 L PEG-ASC *vs* trisulfate (NOCT)^[14], sodium picosulfate plus magnesium citrate (DAYB)^[15] and 2 L PEG (MORA)^[16] have been conducted, showing a non-inferiority respect to comparators.

Nevertheless, despite positive data from RCTs, this product has never been tested in a real-life setting, where patients' characteristics may differ from those of RCTs volunteers, and where the incidence of AEs may even be higher.

MATERIALS AND METHODS

Study design and participants

This is a prospective, multicenter, commercially unfunded, observational study performed across 5 Italian Gastroenterology and Endoscopy units.

All men and women, in- and out-patients aged > 18 years old undergoing a screening, surveillance or diagnostic colonoscopy, after an afternoon-only or afternoon-morning preparation with 4 L-PEG with Selgesse (Alfasigma, Milan, Italy), 2 L-PEG with Moviprep (Norgine, Harefield, United Kingdom) or Clensia (Selgesse, Alfasigma, Milan, Italy) and 1 L-PEG with Plenvu (Norgine, Harefield, United Kingdom) were consecutively enrolled from September 2018 to February 2019.

Patients with known or suspected ileus, gastrointestinal obstruction, bowel perforation, toxic colitis, or megacolon, ongoing severe acute inflammatory bowel disease, previous colonic resection, recent or active gastrointestinal bleeding, pregnancy, allergy or hypersensitivity to the product were excluded.

Techniques

At the time of colonoscopy scheduling, each patient was provided with a form containing the names of the four solutions (Selgesse, Moviprep, Clensia and Plenvu) and, for each, separate instructions for bowel preparation. The solution was independently chosen by the patient based on personal preference, costs, and availability. In the afternoon-morning regimen, all bowel preparations were self-administered taking the first dose the afternoon before the colonoscopy at 6:00 pm \pm 2 h, and the second dose at 5:00 am \pm 2 h the following morning. In the afternoon-only regimen, bowel preparations were entirely consumed the afternoon before the colonoscopy, taking the first dose at 6:00 pm \pm 2 h, and the second dose after an interval of 1-2 h. The 1 L and 2 L-PEG solutions were prepared with 500 mL of additional clear fluids after each dose, and additional clear fluids "ad libitum" were permitted up to two hours before the procedure. A low-fiber diet was recommended in the three days preceding the colonoscopy, while on the day before the colonoscopy, patients were permitted a light breakfast and lunch.

Outcomes and measurement

The primary endpoints of the study were the assessment of bowel cleansing success and high-quality cleansing of the right colon. The secondary endpoints were the evaluation of tolerability, adherence and safety of the different bowel preparations.

Bowel cleansing was assessed through the Boston Bowel Preparation Scale (BBPS), by site unblinded colonoscopists after specific training. A bowel cleansing success was

defined as a total BBPS ≥ 6 with a partial BBPS ≥ 2 in each segment and a high-quality cleansing of the right colon as a partial BBPS = 3.

Adherence was defined as consumption of at least 75% of each dose, while tolerability was evaluated through a semi-quantitative scale with a score ranging from 1 to 10 (1 = lowest rank, 10 = highest rank). Safety was systematically monitored through AEs reporting, collected at the pre-colonoscopy interview.

Statistical analysis

Continuous variables were reported as mean \pm standard deviation, and categorical variables were summarized as frequency and percentage. Independent-samples t-test and χ^2 test were used for comparison of continuous and categorical variables respectively. Pairwise comparisons were adjusted for multiple testing with Benjamini and Hochberg method. Logistic regression models were performed to assess bowel cleansing success and high-quality cleansing of the right colon and to identify the presence of variables associated with outcomes. A generalized linear model was performed to assess tolerability and identify independent predictors. Variables considered into the models were selected through stepwise model selection by Akaike Information Criterion and guided by clinical relevance.

All statistical analyses were performed using R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria) and the statistical review of the study was performed by a biomedical statistician.

Ethics

The study received Ethics Committee approval and was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice. Patients provided written informed consent.

RESULTS

Study population and characteristics

A total of 1566 consecutive patients were evaluated. Of these, 1289 met the inclusion criteria and were enrolled in the study. The main indication for colonoscopy was screening or surveillance for CRC (57.9%). Overall, 52.8% were male, the mean age was 60.5 ± 14.1 , 44.8% of patients were ≥ 65 years old. Hypertension was present in 38.2% of cases, diabetes in 11.2%, obesity in 18.3%, and chronic renal failure in 2.3% of patients.

In the entire cohort, 490 patients performed a 4 L-PEG preparation (Selgesse®), 566 a 2 L-PEG cleansing (Moviprep® or Clensia®) and 233 a 1 L-PEG preparation (Plenvu®). The three populations were homogeneous according to the main characteristics (Table 1).

With regard to the preparation regimen, 62.5% of patients performed an afternoon-only and 37.5% an afternoon-morning (split) preparation, with a similar distribution among the three groups. The mean time between the assumption of the last dose and the beginning of the colonoscopy was 11.9 ± 2.8 in the afternoon-only and 4.9 ± 1.8 h in the split group, respectively.

Bowel cleansing efficacy

Bowel cleansing by BBPS was 6.5 ± 1.5 overall and 6.3 ± 1.5 , 6.2 ± 1.5 , 7.3 ± 1.5 ($P < 0.001$) in the subgroups of 4 L, 2 L and 1 L-PEG preparation, respectively. The average cleansing in the right colon was 1.8 ± 0.6 overall and 1.7 ± 0.6 , 1.6 ± 0.6 , 2.1 ± 0.6 in the subgroups of 4 L, 2 L, and 1 L-PEG ($P < 0.001$) (Figure 1).

Cleansing success and high-quality cleansing of the right colon were assessed in 1248 out of 1289 patients with a complete colonoscopy up to the cecum. An overall cleansing success was achieved in 72.4%, 74.1% and 90.1% ($P < 0.001$ for 1 L vs 2 L; $P < 0.001$ for 1 L vs 4 L) of patients after a 4 L, 2 L and 1 L-PEG preparation (Figure 2A). When assessed by preparation modality, cleansing success was 68.1%, 65.6% and 83.3% ($P = 0.065$ for 1 L vs 2 L; $P = 0.069$ for 1 L vs 4 L) for afternoon-only preparation and 90.3%, 88.3% and 91.9% ($P = 0.84$ for 1 L vs 2 L; $P = 0.84$ for 1 L vs 4 L) for split preparation in the three groups, respectively (Figure 2B and 2C).

A high-quality cleansing of the right colon was achieved in 15.9%, 12.0% and 41.4% ($P < 0.001$ for 1 L vs 2 L; $P < 0.001$ for 1 L vs 4 L) of patients after a 4 L, 2 L and 1 L-PEG preparation (Figure 2D). By subgroup analysis, cleansing success was 10.1%, 9.6% and 14.6% ($P = 0.72$ for 1 L vs 2 L; $P = 0.72$ for 1 L vs 4 L) in the group of afternoon-only preparation and 39.8%, 16.0% and 48.8% ($P < 0.001$ for 1 L vs 2 L; $P = 0.20$ for 1 L vs 4 L) in the group of split preparation in the three groups of 4 L, 2 L and 1 L-PEG preparation (Figure 2E and 2F).

Table 1 Baseline patients' characteristics

	Overall (<i>n</i> = 1289)	4 L PEG (Selgesse®) (<i>n</i> = 490)	2 L PEG (Moviprep® or Clensia®) (<i>n</i> = 566)	1 L PEG (Plenvu®) (<i>n</i> = 233)
Sex, <i>n</i> (%)				
Male	681 (52.8)	268 (54.7)	291 (51.4)	122 (52.4)
Female	608 (47.2)	222 (45.3)	275 (48.6)	111 (47.6)
Age, years, mean ± SD	60.5 ± 14.1	61.1 ± 14.1	60.5 ± 13.5	59.5 ± 15.9
Age group, <i>n</i> (%)				
< 65 yr	708 (55.2)	259 (53.1)	318 (56.5)	131 (56.5)
≥ 65 yr	575 (44.8)	229 (46.9)	245 (43.5)	101 (43.5)
Race group				
White or Caucasian	1254 (97.3%)	479 (97.8%)	550 (97.2%)	225 (96.6%)
Other	35 (2.7%)	11 (2.2%)	16 (2.8%)	8 (3.4%)
Weight, kg, mean ± SD	72.3 ± 14.6	73.3 ± 14.4	72.0 ± 15.4	71.1 ± 13.0
Height, cm, mean ± SD	165.8 ± 9.0	165.9 ± 9.8	165.6 ± 8.5	166.3 ± 8.7
BMI, mean ± SD	26.2 ± 4.6	26.6 ± 4.6	26.2 ± 4.9	25.6 ± 4.0
Obesity	236 (18.3%)	101 (20.6%)	105 (18.6%)	30 (12.9%)
Chronic constipation	215 (16.7%)	84 (17.1%)	101 (17.8%)	30 (12.9%)
Diabetes	144 (11.2%)	68 (13.9%)	62 (11.0%)	14 (6.0%)
Hypertension	492 (38.2%)	218 (44.5%)	223 (39.4%)	51 (21.9%)
Chronic renal failure	30 (2.3%)	11 (2.2%)	12 (2.1%)	7 (3.0%)
Colonoscopy indication, <i>n</i> (%)				
Screening	443 (34.4%)	159 (32.5%)	201 (35.5%)	83 (35.6%)
Surveillance	303 (23.5%)	128 (26.1%)	121 (21.4%)	54 (23.2%)
Diagnostic	543 (42.1%)	203 (41.4%)	244 (43.1%)	96 (41.2%)

PEG: Polyethylene glycol.

Predictors of cleansing success and high-quality cleansing of the right colon

The logistic multiple regression model for overall bowel cleansing success showed that age (OR = 0.99, 95%CI: 0.98-1.00; *P* = 0.024), absence of diabetes (OR = 1.55, 95%CI: 1.01-2.38; *P* = 0.046), adequate cleansing at previous colonoscopy (OR = 2.23, 95%CI: 1.30-3.85; *P* = 0.004), preparation with the 1 L-PEG over the 2 L-PEG (OR = 1.79, 95%CI: 1.04-3.08; *P* = 0.035), afternoon-morning split regimen (OR = 2.52, 95%CI: 1.62-3.92; *P* < 0.001), low-fiber diet for at least 3 days preceding colonoscopy (OR = 2.49, 95%CI: 1.71-3.64; *P* < 0.001), colonoscopy within 5 h after the end of the preparation (OR = 2.35, 95%CI: 1.34-4.10; *P* = 0.003) were independently associated with overall bowel cleansing success (Table 2).

The logistic multiple regression model for high-quality cleansing of the right colon showed that absence of diabetes (OR = 1.93, 95%CI: 1.02-3.65; *P* = 0.043), preparation with the 1 L-PEG both over the 4 L-PEG (OR = 1.58, 95%CI: 1.02-2.44; *P* = 0.041) and the 2 L-PEG (OR = 3.13, 95%CI: 2.08-4.72; *P* < 0.001), preparation with the 4 L PEG over the 2 L-PEG (OR = 1.99, 95%CI: 1.35-2.91; *P* < 0.001), and afternoon-morning split regimen (OR = 3.33, 95%CI: 2.19-5.06; *P* < 0.001) were independently associated with high-quality cleansing of the right colon (Table 3).

Adherence and tolerability

Self-reported adherence to preparation was 95% across all treatment groups and greater for patients undergoing a split *vs* an afternoon-only preparation (98.3% *vs* 93.5; *P* < 0.001).

The adherence was higher, even if not statistically significant, in the group of patients assuming the 1 L-PEG solution compared to the 2 and 4 L solutions and achieved respectively in 98.7%, 95.9% and 93.1% of patients (*P* = 0.357 for 1 L *vs* 2 L; *P* = 0.078 for 1 L *vs* 4 L) (Figure 3A).

Similarly, tolerability was higher for the 1 L preparation compared to the 2 and 4 L-PEG solutions, with an average score of 7.9 ± 1.3 *vs* 7.1 ± 2.0 and 7.3 ± 1.9 (*P* < 0.001 for 1 L *vs* 2 L; *P* < 0.001 for 1 L *vs* 4 L) (Figure 3B). Interestingly, a tolerability rating < 6 was observed in 13.7% and 18.9% of subjects undergoing a 4 and 2 L solution, but

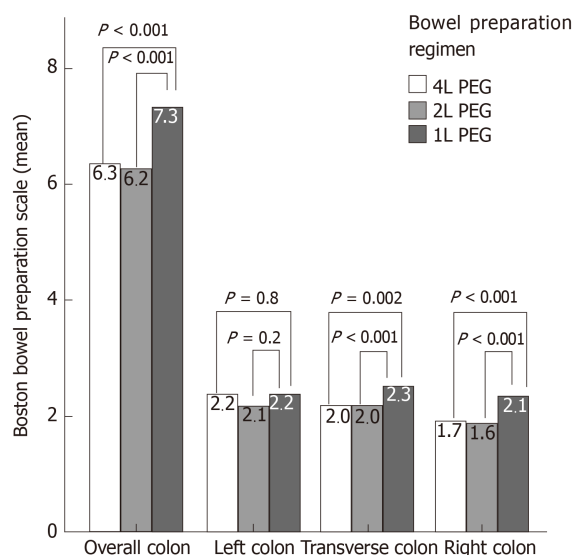


Figure 1 Overall and segmental bowel cleansing scores for 4, 2 and 1 L polyethylene glycol solutions assessed by Boston bowel preparation score. PEG: Polyethylene glycol.

only in 3.5% of patients taking a 1 L preparation ($P < 0.001$).

The multiple regression model showed that female gender (estimate -0.51, 95%CI: -0.72, -0.30; $P < 0.001$), adequate cleansing at previous colonoscopy (estimate 0.65, 95%CI: 0.19-1.10; $P = 0.006$), preparation with the 1 L-PEG both over the 4 L-PEG (estimate 0.57, 95%CI: 0.25-0.90; $P = 0.001$) and the 2 L-PEG (estimate 0.72, 95%CI: 0.41-1.03; $P < 0.001$) and afternoon-morning split regimen (estimate 0.36, 95%CI: 0.06-0.66; $P = 0.019$) were independently associated with tolerability of bowel preparation (Table 4).

Safety

Mild and moderate treatment-emergent AEs (TEAEs), considered to be associated with bowel preparation, were reported in 16.8% of patients overall. By subgroup analysis, TEAEs were reported in 18.4%, 15.7%, and 15.9% ($P = 0.4$) of patients undergoing a 4, 2 and 1 L-PEG preparation, showing no significant difference among the three groups (Supplementary Table 1). The most frequent TEAE was nausea, reported in 5.7% of subjects overall, with a higher incidence of 6.7% in the 4 L group compared to 5.7% and 3.9% of 2 and 1 L groups ($P = 0.4$ for 4 L vs 2 L; $P = 0.1$ for 4 L vs 1 L). Vomit was reported in 2.3% of patients, with a significantly higher incidence in the 1 L compared to the 2 and 4 L groups, respectively of 6.0%, 1.4% and 1.6% ($P = 0.001$ for 4 L vs 1 L; $P < 0.001$ for 4 L vs 2 L). Abdominal pain was present in 2.0% of cases, and mostly in patients undergoing a 4 L preparation compared to the 2 L and the 1 L group, respectively in 3.1%, 1.8% and 0.4% ($P = 0.1$ for 4 L vs 2 L; $P = 0.02$ for 4 L vs 1 L). Dehydration was complained by 0.7% of patients overall, and respectively by 0.8%, 0.4%, and 1.3% of patients in the 4, 2 and 1 L groups ($P = 0.3$). No severe or serious TEAEs were reported.

DISCUSSION

Lower volume preparations may enhance patients' experience in colonoscopy since a reduced liquid intake may be better tolerated. Plenvu has already shown high efficacy in RCTs, with a non-inferiority compared to the higher volume solutions^[14-16]. In this large prospective, multicenter observational study, 1 L PEG-ASC confirmed a higher bowel cleansing effectiveness over the comparators both as average BBPS score overall and in the right colon, both as cleansing success rate overall. The 1 L PEG-ASC solution presented a marginally significant superiority for cleansing success in the subgroup of the afternoon-only preparation, but it did not achieve superiority in the afternoon-morning split subgroup, where it was found to be equivalent to the 4 and 2 L solutions. This is probably secondary to the effectiveness of a split modality, widely shown by several lines of evidence, which may smooth out the differences of efficacy between the three solutions. Nevertheless, the multiple regression model showed Plenvu to be an independent predictor of overall success over the 2 L-PEG preparation and, marginally, over the 4 L-PEG solution.

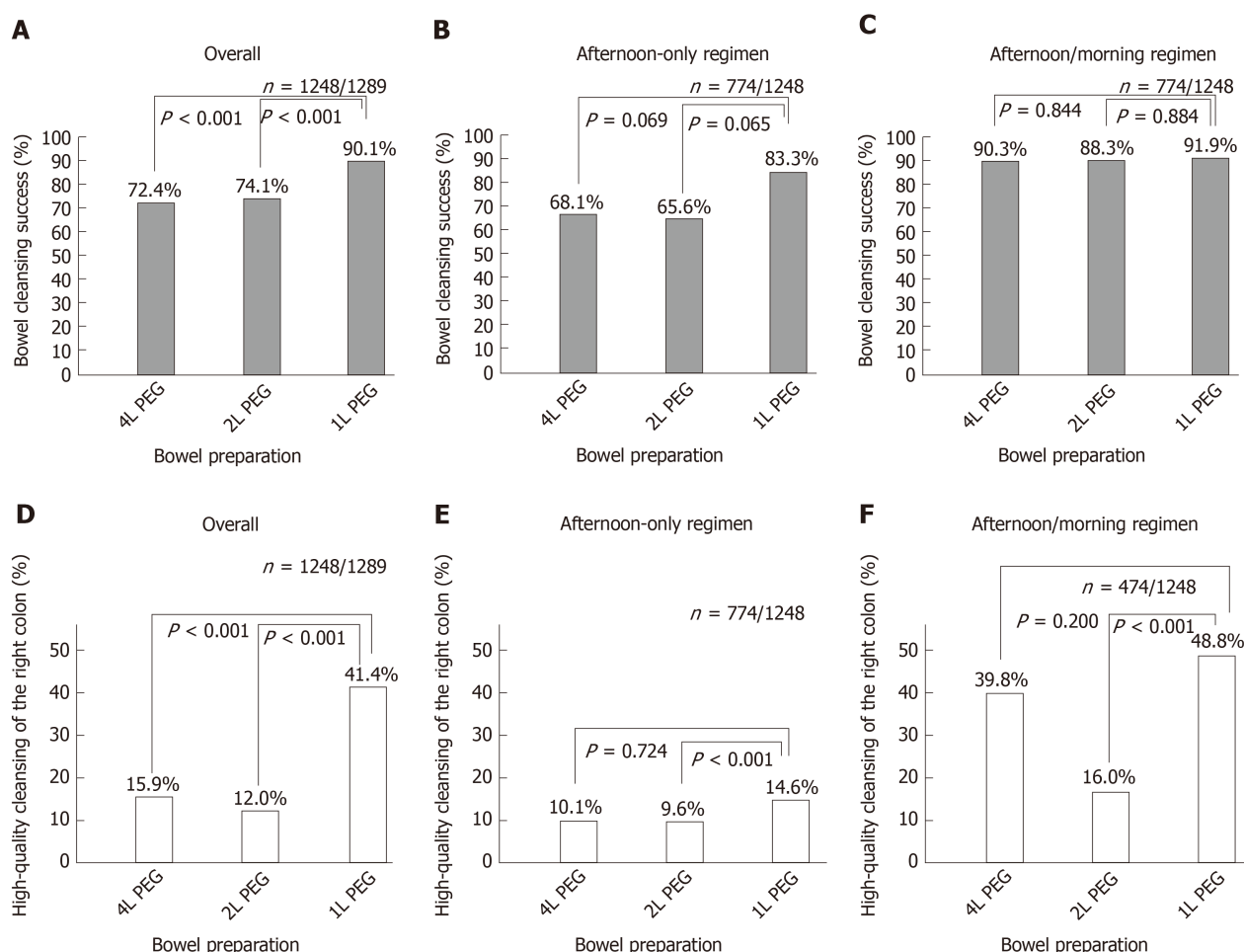


Figure 2 Cleansing success rates and high-quality cleansing of the right colon for 4, 2 and 1 L polyethylene glycol solutions. A-C: Cleansing success rates for 4, 2 and 1 L polyethylene glycol solutions overall (A), in afternoon-only regimen (B) and in afternoon-morning regimen (C); D-F: High-quality cleansing of the right colon for 4, 2 and 1 L polyethylene glycol solutions overall (D), in afternoon-only regimen (E) and in afternoon-morning regimen (F). PEG: Polyethylene glycol.

In addition, Plenvu showed a greater high-quality cleansing of the right colon overall and in the subgroups of afternoon-only and afternoon-morning preparations. This is of primary importance since recent data showed that a high-quality cleansing *vs* adequate cleansing allows doubling the detection rate of high-risk sessile serrated lesions^[4]. Of note, multiple regression model confirmed Plenvu to be an independent predictor of high-quality cleansing of the right colon both over the 2 L and the 4 L preparations.

Our study also showed higher adherence to preparation with Plenvu, compared with other groups, even if this superiority was only marginally significant only over the 4 L-PEG and not significant over the 2 L-PEG. This was predictable, as these are the two groups with the most significant difference in terms of total volume (3 L), while the small volume difference between 1 and 2 L solutions can justify a comparable adherence.

Similarly, individual tolerability, based on the patients' judgment, was higher for Plenvu than for other solutions. To note, insufficient tolerability, defined as a score < 6, was reported in only a small minority of patients undergoing a 1 L-PEG preparation (3.9%), compared to a higher percentage of patients undergoing a 4 L (13.6%) and a 2 L (18.9%) solution.

Finally, multiple regression model, confirmed Plenvu to be an independent predictor of tolerability of bowel preparation both over the 2 L and the 4 L solutions. In particular, the combination of 1 L-PEG solution and of a split regimen showed to provide the highest tolerability. These data are of particular importance since they confirm the theoretical assumption that a lower volume solution is also well tolerated.

Overall, the investigated solutions presented a good safety profile in the absence of significant differences in TEAEs across the three groups. However, the type of AEs was different. Nausea was slightly higher in the group of patients undergoing a 4 L solution, compared to the other two groups, even if this difference was not statistically significant. Similarly, abdominal pain presented a higher incidence in the

Table 2 Logistics multiple regression model estimates for overall cleansing success

Predictors	Odds ratios	95%CI	P value
Female gender	1.00	0.75-1.34	0.975
Age, yr	0.99	0.98-1.00	0.024
BMI	0.98	0.95-1.01	0.217
Diabetes (absence)	1.55	1.01-2.38	0.046
Hypertension	1.35	0.97-1.89	0.077
Chronic constipation (absence)	1.11	0.77-1.61	0.578
Previous adequate cleansing	2.23	1.30-3.85	0.004
Preparation (ref = 4 L PEG)			
2 L PEG	0.89	0.66-1.20	0.458
1 L PEG	1.60	0.91-2.80	0.099
Preparation (ref = 2 L PEG)			
4 L PEG	1.12	0.83-1.51	0.458
1 L PEG	1.79	1.04-3.08	0.035
Afternoon-morning split regimen	2.52	1.62-3.92	< 0.001
Low fiber diet \geq 3 d	2.49	1.71-3.64	< 0.001
Time lag \leq 5 h	2.35	1.34-4.10	0.003

BMI: Body mass index; PEG: Polyethylene glycol.

group of the 4 L-PEG, which was significant only over the 1 L group. This difference is probably secondary to the large volume of the 4 L preparation, over the lower-volume ones, which can cause intestinal distension symptoms.

Conversely, vomit was more frequent for the 1 L solution, with a significantly higher incidence over the other two groups. This higher incidence of vomiting after the consumption of Plenvu is probably secondary to the greater amount of ascorbate, which is present in the second dose. Nevertheless, it is crucial to remark that the intensity of vomit was mild and not able to compromise the tolerability of bowel preparation. As a matter of fact, in our study, Plenvu was the most tolerated solution and it was also found to be an independent predictor of tolerability.

Dehydration symptoms were reported in less than 1.5% of patients, with a comparable incidence between the three groups, and no severe or serious AEs or deaths were registered in the entire cohort. Given the observational nature of our study, an assessment of blood electrolyte or creatinine was not feasible. Despite this, no clinical event attributable to electrolyte imbalance or dehydration was observed in any patient.

This is, at best of our knowledge, the first study assessing the effectiveness and tolerability of Plenvu in the real-life. The major strengths of this study are its prospective and multicenter design, the presence of a large sample size and the comparison of the 1 L-PEG preparation with both the 2 and 4 L-PEG solutions. Nevertheless, this study is limited by a few relevant factors. First of all, the absence of randomization, which guarantees the similarity across the treatment groups, and avoids exposure to potential bias, among all the “allocation of intervention bias”.

Nevertheless, the absence of randomization is secondary to the nature of the study that was explicitly intended to be observational. This provides the advantage to evaluate the effectiveness and the tolerability of the product in conditions that are far from the selectivity of the RCTs and closer to real life.

Secondly, the absence of blinding between site colonoscopists and type of bowel preparation performed by patients. This was mainly due to overt differences of treatments, depending on patient characteristics and indication, which did not make a blinding design feasible. In fact, in some centers, colonoscopists are aware that some categories of patients (*e.g.*, inpatients and patients undergoing screening), received only one specific preparation directly provided by the hospital.

Nevertheless, the absence of blinding exposes to additional potential biases, primarily to the observer expectation bias, since the knowledge of the hypotheses can influence the observer to stretch out for the product being tested, compared to the reference standard products.

Finally, the cleansing success rates observed in this study were suboptimal. This may depend on the demographic characteristics of the study population with a relevant proportion of elderly, inpatients and patients with comorbidities, factors that

Table 3 Logistics multiple regression model estimates for high-quality cleansing of the right colon

Predictors	Odds ratios	95%CI	P value
Female Gender	1.25	0.91-1.72	0.169
Age, yr	1.00	0.99-1.01	0.927
BMI	0.98	0.95-1.02	0.336
Diabetes (absence)	1.93	1.02-3.65	0.043
Hypertension	1.00	0.69-1.44	0.983
Chronic constipation (absence)	0.70	0.47-1.04	0.080
Previous adequate cleansing	1.06	0.52-2.18	0.863
Preparation (ref = 4 L PEG)			
2 L PEG	0.50	0.34-0.74	< 0.001
1 L PEG	1.58	1.02-2.44	0.041
Preparation (ref = 2 L PEG)			
4 L PEG	1.99	1.35-2.91	< 0.001
1 L PEG	3.13	2.08-4.72	< 0.001
Afternoon-morning split regimen	3.33	2.19-5.06	< 0.001
Low fiber diet \geq 3 d	0.99	0.63-1.56	0.967
Time lag \leq 5 h	1.22	0.81-1.83	0.337

BMI: Body mass index; PEG: Polyethylene glycol.

may affect the quality of the bowel preparation.

Concerning the scales used for the assessment of the outcomes, we used the BBPS as it is the most widely validated and the only one that significantly correlates with polyp detection and surveillance intervals^[17]. With regards to tolerability, we used a semi-quantitative scale with a score ranging from 1 to 10 by the judgment of the patients, since a validated scale for the assessment of bowel preparation tolerability is not currently available. In the NOCT study only, the Bowel Cleansing Impact Review (BOCLIR) was used^[18]. Nevertheless, this is a complex score that features 34 items, and external validation of the score is absent.

In conclusion, results from this study support the effectiveness and tolerability of very low-volume preparation for colonoscopy and also show it is an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability. In the future, this very-low-volume solution will be useful to improve the quality and tolerability of bowel preparation increasing, at the same time, the adherence to CRC screening and surveillance programs.

Table 4 Multiple regression model estimates for tolerability of bowel preparation

Predictors	Estimates	95%CI	P value
Female Gender	-0.51	-0.72, -0.30	< 0.001
Age, yr	-0.00	-0.01-0.01	0.858
BMI	-0.00	-0.02-0.02	0.889
Diabetes (absence)	0.12	-0.22-0.46	0.489
Hypertension	0.25	-0.04-0.49	0.084
Chronic constipation (absence)	-0.20	-0.48-0.07	0.153
Previous adequate cleansing	0.65	0.19-1.10	0.006
Preparation (ref = 4 L PEG)			
2 L PEG	-0.15	-0.37-0.08	0.204
1 L PEG	0.57	0.25-0.90	0.001
Preparation (ref = 2 L PEG)			
4 L PEG	0.15	-0.08-0.37	0.204
1 L PEG	0.72	0.41-1.03	< 0.001
Afternoon-morning split regimen	0.36	0.06-0.66	0.019
Low fiber diet ≥ 3 d	0.16	-0.09-0.41	0.219

BMI: Body mass index; PEG: Polyethylene glycol.

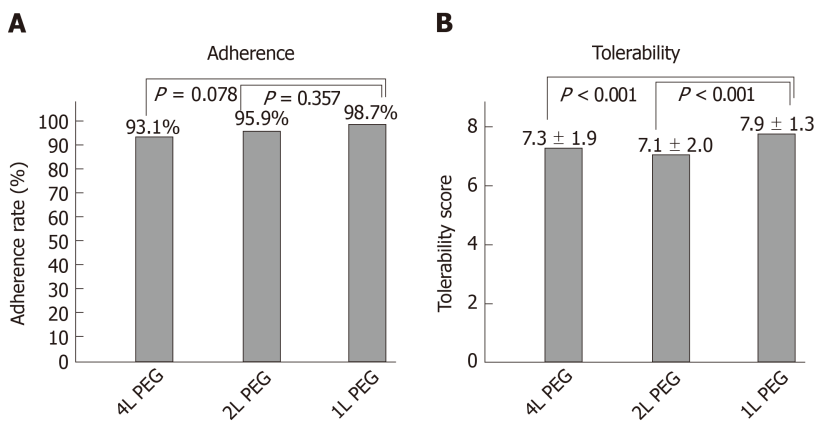


Figure 3 Adherence and tolerability of 4, 2 and 1 L polyethylene glycol solutions. A: Adherence of 4, 2 and 1 L polyethylene glycol solutions; B: Tolerability of 4, 2 and 1 L polyethylene glycol solutions. PEG: Polyethylene glycol.

ARTICLE HIGHLIGHTS

Research background

The effectiveness of colonoscopy strictly depends on adequate bowel cleansing. Recently, a 1 L polyethylene glycol plus ascorbate (PEG-ASC) solution has been introduced on the evidence of three phase-3 randomized controlled trials.

Research motivation

The 1 L PEG-ASC solution has never been tested in a real-life setting, where patients' characteristics may differ from those of randomized controlled trials volunteers, and where the incidence of adverse events (AEs) may even be higher.

Research objectives

In this study, we aimed to assess the effectiveness and tolerability of the 1 L preparation compared to 4 L and 2 L-PEG solutions in a real-life setting.

Research methods

Patients undergoing a screening or diagnostic colonoscopy after a 4, 2 or 1 L PEG preparation, were consecutively enrolled in 5 Italian centers. Bowel cleansing was assessed through the Boston Bowel Preparation Scale (BBPS). Adherence was defined as consumption of at least 75% of each dose, while tolerability was evaluated through a semi-quantitative scale. Safety was systematically monitored through AEs reporting.

Research results

Bowel cleansing by Boston Bowel Preparation Scale was 6.5 ± 1.5 overall and 6.3 ± 1.5 , 6.2 ± 1.5 , 7.3 ± 1.5 in the subgroups of 4 L, 2 L and 1 L-PEG preparation, respectively. Cleansing success was achieved in 72.4%, 74.1% and 90.1%, while a high-quality cleansing of the right colon in 15.9%, 12.0% and 41.4% for 4 L, 2 L and 1 L-PEG preparation groups, respectively. The 1 L preparation was the most tolerated compared to the 2 and 4 L-PEG solutions in the absence of serious AEs within any of the three groups. Multiple regression models confirmed 1 L PEG-ASC preparation as an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability.

Research conclusions

The effectiveness and tolerability of 1 L PEG-ASC show that it is an independent predictor of overall cleansing success, high-quality cleansing of the right colon and of tolerability.

Research perspectives

This very-low-volume solution will be useful to improve the quality and tolerability of bowel preparation increasing, and the adherence to colorectal cancer screening and surveillance programs.

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Randomized Clinical Trial

Retrograde inspection vs standard forward view for the detection of colorectal adenomas during colonoscopy: A back-to-back randomized clinical trial

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Abstract

BACKGROUND

The adenoma detection rate (ADR) is inversely associated with the incidence of interval colorectal cancer and serves as a benchmark quality criterion during screening colonoscopy. However, adenoma miss rates reach up to 26% and studies have shown that a second inspection of the right colon in retroflected view (RFV) can increase ADR.

AIM

To assess whether inspection of the whole colon in RFV compared to standard forward view (SFV) can increase ADR.

METHODS

Patients presenting for screening or surveillance colonoscopy were invited to participate in this randomized controlled trial and randomized into two arms. In RFV arm colonoscopy was initially performed with SFV, followed by a second inspection of the whole colon in RFV. In the SFV arm first withdrawal was performed with SFV, followed by a second inspection of the whole colon again with SFV. Number, size and morphology of polyps found during first and second inspection in each colonic segment were recorded and all polyps were removed and sent for histopathology in separate containers.

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RESULTS

Two hundred and five patients were randomly assigned to the RFV ($n = 101$) and SFV ($n = 104$) arm. In the RFV arm, both polyp detection rate (PDR) and ADR were increased under second inspection in RFV (PDR 1st SFV: 39.8%, PDR 2nd RFV: 46.6%; ADR 1st SFV: 35.2%, ADR 2nd RFV: 42%). Likewise, in the SFV arm, PDR and ADR were increased under second inspection (PDR 1st SFV: 37.5%, PDR 2nd SFV: 46.6%; ADR 1st SFV: 34.1%, ADR 2nd SFV: 44.3%) with no significant differences in ADR and PDR between the SFV and RFV arm. Mean number of adenomas per patient (APP) was increased in the RFV and SFV (APP RFV arm: 1st SFV: 1.71; 2nd RFV: 2.38; APP SFV arm: 1st SFV: 1.83, 2nd SFV: 2.2). The majority of adenomas additionally found during second inspection in RFV or in SFV were located in the transverse and left-sided colon and were > 5 mm in size.

CONCLUSION

Second inspection of the whole colon leads to increased adenoma detection with no differences between SFV and RFV. Hence, increased detection is most likely a feature of the second inspection itself but not of the inspection mode.

Key words: Colorectal cancer; Adenoma; Adenoma detection rate; Colonoscopy

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Core Tip: This is the first study to systematically assess the effect of an additional retrograde inspection of the whole colon on adenoma detection rate compared to a second inspection in standard forward view. Our results show that both, additional inspection of the entire colon in retroflexion as well as in forward view leads to an increased adenoma detection rate with no differences between retrograde and forward inspection. Further, the majority of adenomas additionally found during second inspection in retroflexion or in forward view were located in the transverse and left-sided colon and were > 5 mm in size. These results clearly suggest that increased adenoma detection is most likely a feature of the second inspection itself but not of the inspection mode.

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INTRODUCTION

The adenoma detection rate (ADR), defined as the percentage of individuals undergoing screening colonoscopy in which at least one adenoma is found, is inversely associated with the incidence in interval colorectal cancers (CRC). In this regard, it has been shown that a 1% increase of the ADR results in a decrease of interval CRC incidence by 3%^[1]. Further, as shown in prospective long-term follow-up studies, removal of adenomatous polyps during colonoscopy reduces the incidence and mortality of CRC^[2,3] and large cohort studies have shown that the CRC mortality can be reduced up to 70% by screening colonoscopy^[4]. Based on these considerations, ADR has been implemented as a key benchmark criterion to assess quality during screening colonoscopy in clinical practice guideline across the globe^[5,6]. However, at the same time, colonic neoplasia can frequently be missed during screening colonoscopy with miss rates for adenomas reaching up to 26%, as shown in a recent meta-analysis^[7]. Several factors are considered to attribute to these high miss rates, among them human error and blind spots as major factors. Among the various means to limit miss rates, simple modification of standard colonoscopy such as change of patients' position, appliance of abdominal compression or a second inspection of the colon in either standard forward view (SFV) or retroflexed view (RFV) have shown to improve ADR^[8-12]. The latter has been addressed by numerous studies and although it

has been shown that a second inspection in SFV or RFV can significantly increase ADR, these studies have utilized second inspection predominantly in the right sided colon.

Within this study we assessed whether additional inspection of the whole colon in retroflexion compared to a second inspection in standard forward view can increase ADR.

MATERIALS AND METHODS

Patients

The study was approved by the local ethics committee (IRB No. 366_18B) and was performed in accordance with the declaration of Helsinki. The study was registered at ClinicalTrials.gov under the following ID: NCT04107376. Patients presenting for colonoscopy in the Ludwig Demling Endoscopy Center of Excellence were included in this prospective back-to-back randomized controlled trial under the following inclusion criteria: Screening or surveillance colonoscopy, colonoscopy for the work-up of abdominal pain and/or change in bowel habits. Exclusion criteria were as followed: Inflammatory bowel diseases, known polyps or referral for polypectomy, presence of coagulopathy, inadequate bowel preparation with a total Boston Bowel Preparation Score (BBPS) < 6 or the presence of a segment with a BBPS < 2. Patients with diagnosis of CRC during colonoscopy were also excluded. Prior to study inclusion written informed consent was obtained from all participating subjects, minors were excluded. **Figure 1** provides an overview of the screened and studied patient cohort according to the 'CONSORT' statement for randomized trials^[13].

Randomization and colonoscopy procedure

All patients received bowel preparation with low-volume PEG-based bowel lavage in a split dose regimen. On the day of endoscopy, patients were randomized using sealed envelopes into the following two arms (**Figure 2**): (1) RFV arm: Colonoscopy was initially performed with SFV, followed by a second inspection of the whole colon in RFV; and (2) SFV arm: Colonoscopy was initially performed with SFV, followed by a second inspection of the whole colon again with SFV. Allocation was concealed using opaque envelopes until just before initiation of the procedure. To systematically assess the influence of the inspection modality on polyp and adenoma detection within the different segments of the colon, the colon was divided into the following three segments: Caecum and ascending colon, transverse colon, descending and sigmoid colon (**Figure 3**). Every segment was first inspected with SFV followed by inspection of the same segment with either second SFV (SFV Arm) or in retroflexion (RFV arm) colonoscopy with first SFV. In the RFV arm a dedicated small bending HD colonoscope with an outer diameter of 11.6 mm (RetroView EC-34 i10T, Pentax Medical, Tokyo, Japan) while in the SFV arm, a regular HD colonoscope with an outer diameter of 13.2 mm was used (i10F2, Pentax Medical, Tokyo, Japan). Insertion time as well as withdrawal times in every segment under either SFV or RFV were recorded using a stop watch. During further cleaning of the colon, polyp assessment (morphology and size) and polyp removal, the stop watched was paused. Morphology of polyp and adenomas in each segment were assessed using the "Paris" criteria^[14], polyp size was evaluated against an open biopsy forceps with a diameter of 7 mm. All polyps and adenomas found during colonoscopy were removed using either cold- or hot-snare polypectomy at the discretion of the endoscopist, formalin fixed in separate containers after removal and analysed by experienced gastrointestinal pathologists.

Endpoints

The primary endpoint was the ADR, defined as the proportion of patients with at least one adenoma. Secondary endpoints were the polyp detection rate, the mean number of adenomas per patient, the withdrawal time and the success of complete inspection of the whole colon in retroflexion.

Statistical analysis

All data are presented as mean, median, SD and range, as indicated in the respective figures and tables. Grouped continuous data were compared using the Mann-Whitney U-test. Intergroup and categorical comparisons were made using the χ^2 and Fisher's exact tests. A two-sided $P < 0.05$ was considered to be significant. The exact value was reported with P between 0.05 and 0.001, whereas $P < 0.001$ was reported for values below it. Interobserver variability in the ADR was calculated using "Kappa" statistics. The statistics were processed using the SPSS version 19 (SPSS Inc, Chicago, IL, United States).

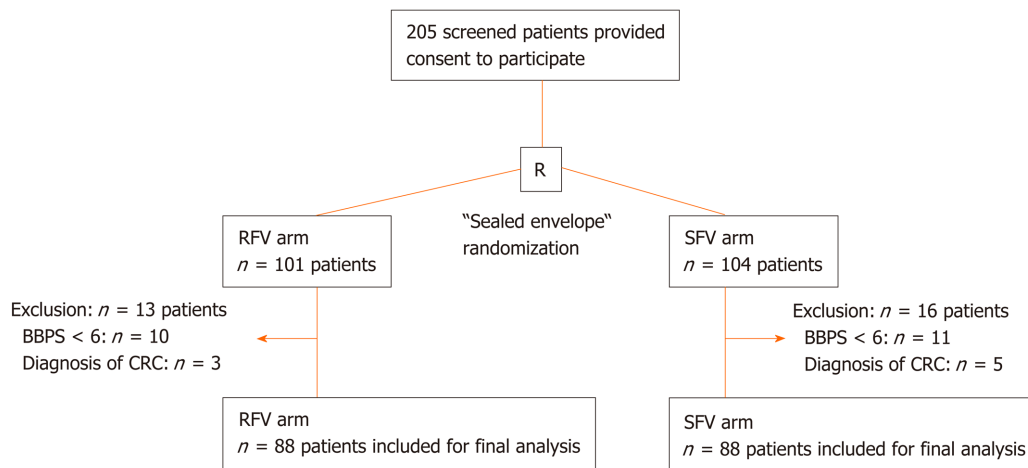


Figure 1 Flowchart of patients included in the study, presented according to the CONSORT statement. R: Randomization; RFV: Retroflected view; SFV: Standard forward view; CRC: colorectal cancer.

RESULTS

Clinical characteristics of the patient cohort

A total of 205 patients consented to participate in the study and were randomized into the SFV arm ($n = 104$) and the RFV arm ($n = 101$). Of these, 29 patients had to be excluded due to inadequate bowel preparation (SFV arm: $n = 10$, RFV arm: $n = 11$) or the diagnosis of CRC (SFV arm: $n = 5$, RFV arm: $n = 3$) during endoscopy. Therefore, 176 patients were included in the final analysis (SFV arm: $n = 88$, RFV arm: $n = 88$). A flowchart of the patient inclusion according to the 'CONSORT' statement^[13] is shown in Figure 1.

Baseline demographics of the patients randomized to the SFV and RFV arm showed no statistically significant differences: As shown in Table 1, in the SFV arm, 45% of patients were female (39 out of 88) with a mean age of 59.3 ± 15.1 years (range 18-86) whereas in the RFV arm, 42% of patients included for final analyses were female (37 out of 88) with a mean age of 59.9 ± 15.5 years (range 20-88). Withdrawal times showed no significant differences between the different inspection modalities in each colonic segment and in each study arm (Table 1).

In the RFV arm, second inspection of each colonic segment in retroflexion was possible in 86 out of 88 patients (97.7%). In one patient retroflexion in the caecum was not possible due to severe looping, in the other patient inspection of the sigmoid in retroflexion was incomplete due to severe angulation. In both patients, the second examination was then performed with standard forward view.

In the RFV arm, PDR was 39.8% after the first inspection with standard forward view and increased to 46.6% after second inspection of the whole colon in retroflexion. Baseline ADR after first inspection in SFV was 35.2% in the RFV arm (Table 2). Second inspection of each colonic segment in retroflexion led to an additional detection of adenomas in six patients, which had no adenomas during first inspection with standard forward view; therefore, ADR was increased to 42% under second inspection of the colon in retroflexion (Table 2). Interobserver variability in the ADR between the six endoscopists showed substantial agreement during both, first and second withdrawal (first withdrawal: $\kappa = 0.73$, second withdrawal: $\kappa = 0.69$) in the RFV arm. Mean number of adenomas was 1.71 per patient after first inspection with standard forward view and increased to a mean of 2.38 adenomas per patient after second inspection of the colon in retroflexion in the RFV arm. Importantly, among the 35 in retroflexion additionally detected adenomas, the majority were greater than 5 mm (19/35, 54%), sessile or flat elevated (Paris Is: 18/35, 51%; Paris Ila: 15/35, 43%) and two sessile serrated adenomas (Table 3), thereby indicating that the adenomas additionally detected in retroflexion were indeed clinically relevant lesions.

In the SFV arm, PDR after first inspection in SFV was 37.5% and increased to 46.6% after second inspection of the colon again in SFV. Baseline ADR after first inspection with SFV was 34.1% in the SFV arm. Second inspection of each colonic segment in SFV led to the detection of adenomas in additional nine patients in which no adenomas had been detected during first inspection with standard forward view. Thus, second inspection with standard forward view led to an increase in ADR to 44.3% in the SFV arm (Table 2). Interobserver variability in the ADR between the six endoscopists again showed substantial agreement during both, first and second

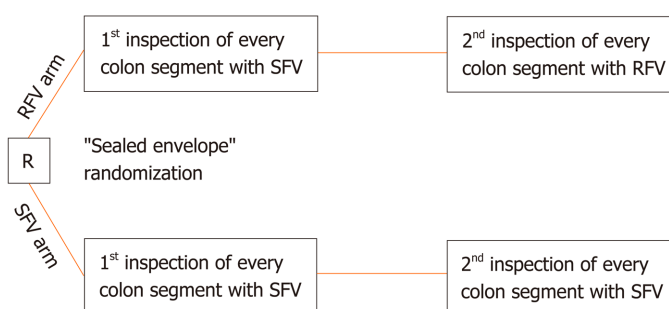


Figure 2 Randomization of the included patients and inspection modes in the retroflected view and standard forward view arm. R: Randomization; RFV: Retroflected view; SFV: Standard forward view.

withdrawal (first withdrawal: $\kappa = 0.75$, second withdrawal: $\kappa = 0.71$) in the RFV arm.

Mean adenoma per patient rate in the SFV arm was 1.83 adenomas per patient after first inspection in SFV mode and increased to a mean of 2.20 adenomas after second inspection in SFV view. Among the 31 adenomas additionally detected during second forward inspection, the majority were greater than 5 mm (19/31, 61%), sessile or flat elevated (Paris Is: 9/31, 29%; Paris IIa: 16/31, 52%). Further, histology of additionally found lesions during second SFV inspection showed two adenomas with high-grade dysplasia and two sessile serrated adenomas, one of which exhibited dysplasia (Table 3).

DISCUSSION

As summarized in a recent systematic review and meta-analysis, additional retrograde inspection of the right colon after first inspection in standard forward view is not only a safe but also effective procedure that can significantly increase ADR^[15]. In this regard, it has been shown from a total of 3660 colonoscopies that standard colonoscopy with additional right-sided retroflexion compared to conventional colonoscopy alone, that a pooled per-adenoma miss rate of 17% is present in the right colon by not performing right colon retroflexion^[15]. At the same time, several studies have shown that a second inspection of the right-sided colon in standard forward view can likewise increase ADR^[11,16] and the increase of ADR through a second inspection of the right sided colon with a second forward inspection has been confirmed by a recent meta-analysis^[17].

This has led to the theory that the increase of ADR is more likely attributable to the second inspection itself and the associated increase in withdrawal time but not a function of the mode of inspection (SFV *vs* RFV) during second withdrawal. Further evidence to support this comes from another recent meta-analysis comparing the diagnostic yield of a second forward view compared with retroflexion examination for the detection of right-sided adenomas^[18]. As shown in this report, a second forward view and retroflected view of the right side of the colon were both associated with improvement in ADR and importantly, when the adenoma miss rate between the second forward view and retroflexion were compared, no statistically significant difference was found^[18]. In their totality, these studies suggest that the key aspect for increasing the ADR in the right sided colon is the second inspection itself but not the mode of inspection.

However, all of the above-mentioned studies were limited to studying the effects of a second inspection only in the right-sided colon. To date, data on a potential increase of ADR through second inspection of the transverse and left-sided colon and especially the comparison of a second inspection in SFV and RFV in other segments than the right colon are completely missing to date.

To fill this gap, we set off to systematically assess the effects of a second retrograde inspection of the whole colon on ADR. In order to control for the effect of second inspection itself, we designed this as a randomized back-to-back study in which in both arms, colonoscopy was initially performed with standard forward view and followed by a second inspection of the whole colon in either retroflected view (RFV arm) or a second inspection with further standard forward view (SFV arm). As shown by the withdrawal time in the two arms, inspection times were virtually identically between the different inspection modalities in each colonic segment, thereby suggesting that inspection times were well controlled and therefore most likely do not represent a significant bias for primary outcome in the two arms of our study. Our

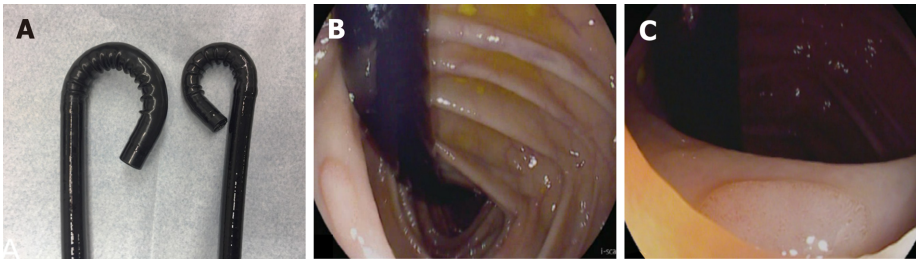


Figure 3 Retrograde inspection of the whole colon. A: For retrograde inspection of the whole colon, a dedicated high definition colonoscope with an outer diameter of 11.6 mm and a bending radius allowing for $> 210^\circ$ deflection was used (right: RetroView EC-34 i10T; left: standard colonoscope i10F2, both from Pentax Medical, Tokyo, Japan); B and C: Detection of a sessile adenoma behind a colonic fold in retroflexion in the transverse colon in the retroflected view arm.

results clearly show that both, second inspection of the colon with RFV as well as second inspection SFV leads to an increase of ADR by 7% to 10% with no significant differences between second withdrawal in RFV or SFV. Therefore, these data not only corroborate the findings from similar studies in the right colon, but also extends this to the transverse and left-sided colon. Remarkably, out of 35 (RFV) and 31 (SFV) polyps additionally detected during second inspection of the whole colon, 2/3 of lesions were located in the transverse and left-sided colon, thereby clearly showing that a second inspection also of these segments is an effective procedure to further detect a substantial number of polyps. Further, as shown by the polyp characteristics, adenomas found during second inspection in either SFV or RFV were not only diminutive or small lesions, but also clinically relevant lesions such as adenomas > 10 mm, adenomas with advanced histology such as HGIEN or SSAs. In their totality, these data support the concept that second inspection of the whole colon in either retroflexion or standard forward view is an easy but effective procedure for increased detection of relevant pathology throughout the colon. Recently, it has been verified on the level of a meta-analysis that serum CrP levels are positively associated with advanced colorectal adenoma risk and subgroup and stratified analyses revealed a potential influence of smoking status and aspirin use on the association between CRP levels and colorectal adenoma risk^[19]. Hence, it can be envisioned that in the future second inspection of the whole colon in either forward or retroflected view might represent an easy and effective means to increase detection of clinically relevant lesions especially in patients at risk for developing advanced adenomas as identified by Godos and co-workers^[19].

At the same time, limitations of the current study also need to be addressed. Although the study was designed as a randomized controlled back-to-back study, with its setting at a single academic center, results might not be directly applicable to the community setting. However, this aspect might be mitigated by the fact that five different endoscopists performed colonoscopies in the current study. Nevertheless, it seems clear that larger multi-center studies are highly warranted to further corroborate our findings.

In summary, our study shows that second inspection of the whole colon leads to increased adenoma detection with additional and clinically relevant lesions found throughout the entire colon. Further, our results clearly show that re-inspection of the colon in retroflexion is not superior over a second examination in standard forward view, thereby suggesting that the increase in adenoma detection is most likely attributable to the second inspection itself and independent of the inspection mode. Hence, second inspection of the colon can be considered as an easy approach to increase ADR and effectiveness of screening or surveillance colonoscopy.

Table 1 Patient characteristics and withdrawal times in the standard forward view and retroflected view arm

	SFV arm		RFV arm	
Patients, <i>n</i> (m/f)	88 (39/49)		88 (37/51)	
Age, yr				
mean	59.3 ± 15.2		59.9 ± 15.6	
range	18-86		20-88	
Withdrawal time (min)	1 st SFV	2 nd SFV	1 st RFV	2 nd RFV
Cecum & ascending colon	1:51 (1:05-2:27)	1:47 (0:42-2:25)	1:53 (1:27-2:17)	1:52 (0:50-2:17)
Transverse colon	1:46 (0:59-2:54)	1:49 (1:05-2:41)	1:50 (1:20-2:50)	1:44 (0:30-2:49)
Descending & sigmoid colon	2:36 (1:58-4:22)	2:30 (1:00-3:41)	2:23 (1:23-2:54)	2:29 (1:31-2:59)

RFV: Retroflected view; SFV: Standard forward view.

Table 2 Polyp detection rates and adenoma detection rates after first and second inspection in the standard forward view and retroflected view arm

	SFV arm	RFV arm	<i>P</i> value
PDR			
1 st Inspection	33/88 = 37.5%	35/88 = 39.8%	0.870
2 nd Inspection	41/88 = 46.6%	41/88 = 46.6%	1.000
ADR			
1 st inspection	30/88 = 34.1%	31/88 = 35.2%	1.000
2 nd inspection	39/88 = 44.3%	37/88 = 42%	0.8791

RFV: Retroflected view; SFV: Standard forward view; PDR: Polyp detection rate; ADR: Adenoma detection rate.

Table 3 Characteristics of the polyps detected during first and second inspection in the standard forward view and retroflected view arm

	SFV arm		RFV arm	
	1 st SFV	2 nd SFV	1 st SFV	2 nd RFV
Adenoma size				
< 5 mm	29	12	14	16
5-10 mm	19	16	31	17
> 10 mm	7	3	8	2
Adenoma localization				
Cecum and ascending colon	18	9	21	11
Transverse colon	11	6	9	11
Descending and sigmoid colon	26	16	23	13
Histology				
LGIE	53	27	48	33
HGIE	1	2	2	0
SSA wo dysplasia	1	1	2	2
SSA with dysplasia	0	1	1	0
Paris classification				
Is	21	9	28	18
Ip	2	1	0	0
Ila	28	16	18	15
Ilb	4	5	7	2

RFV: Retroflected view; SFV: Standard forward view; LGIE: Low grade intraepithelial neoplasia; HGIE: High grade intraepithelial neoplasia; SSA: Sessile serrated adenoma.

ARTICLE HIGHLIGHTS

Research background

Due to its inverse association with the incidence of interval colorectal cancer (CRC), the adenoma detection rate (ADR) serves as a key benchmark criterion for quality assessment in screening and surveillance colonoscopy worldwide. In this regard it has been shown that a 1% increase of the ADR results in a decrease of interval CRC incidence by 3%. At the same time, colonic neoplasia can frequently be missed during screening colonoscopy with miss rates for adenomas reaching up to 26% and human error as well as blind spots are considered the major factors contributing to these high miss rates.

Research motivation

Among the various means to limit miss rates, simple modification of standard colonoscopy such as change of patients' position, appliance of abdominal compression or a second inspection of the colon in either standard forward view (SFV) or retroflected view (RFV) have shown to improve ADR. The latter has been addressed by several studies and although it has been shown that a second inspection in SFV or RFV can significantly increase ADR, these studies have utilized second inspection predominantly in the right sided colon. Within this study we therefore analyzed whether additional inspection of the whole colon in RFV can increase ADR compared to an additional inspection in SFV.

Research objectives

In this study we aim to assess whether inspection of the whole colon in RFV compared to standard forward view SFV can increase ADR.

Research methods

To address the question whether additional retrograde inspection of the whole colon can significantly increase ADR, we designed this study as prospective randomized back-to-back trial, in which patients were randomized used sealed envelopes into the following arms: (1) RFV arm: Colonoscopy was initially performed with SFV, followed by a second inspection of the whole colon in RFV; and (2) SFV arm: Colonoscopy was initially performed with SFV, followed by a second inspection of the whole colon again with SFV. Insertion time as well as withdrawal times in every segment under either SFV or RFV were recorded using a stop watch and all polyps and adenomas found were removed using either cold- or hot-snare polypectomy.

Research results

205 patients were randomly assigned to the RFV ($n = 101$) and SFV ($n = 104$) arm. In the RFV arm, both polyp detection rate (PDR) and ADR were increased under second inspection in RFV. Likewise, in the SFV arm, PDR and ADR also increased under second inspection and importantly, no significant differences in ADR and PDR between the SFV and RFV arm were found. Consistent with this, the mean number of adenomas per patient (APP) was increased in both, the RFV and SFV (APP RFV arm: 1st SFV: 1.71; 2nd RFV: 2.38; APP SFV arm: 1st SFV: 1.83, 2nd SFV: 2.2). The majority of adenomas additionally found during second inspection in RFV or in SFV were located in the transverse and left-sided colon and were > 5 mm in size.

Research conclusions

Second inspection of the whole colon in either standard forward view or retroflected view leads to increased adenoma detection with no significant differences between these two inspections modalities. Hence, increased detection is most likely a feature of the second inspection itself but not of the inspection mode.

Research perspectives

A second inspection of the colon in either standard forward view or retroflected view can be considered as an easy approach to increase ADR. Further large multi-center studies should assess whether this approach can increase effectiveness of screening or surveillance colonoscopy and reduce CRC mortality.

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Severe steroid refractory gastritis induced by Nivolumab: A case report

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Abstract

BACKGROUND

Immune checkpoint inhibitors are widely used for treatment of many advanced malignancies. Lower gastrointestinal (GI) side effects, such as diarrhea and colitis, are common, but upper GI side effects are rarely reported. Consequently, the correct treatment of upper GI adverse events has been less frequently described.

CASE SUMMARY

We describe a case of a 16-year-old woman with stage IIIB malignant melanoma treated with adjuvant monotherapy using Nivolumab. The patient developed severe gastritis after six series of Nivolumab with weight loss, nausea, and vomiting. There was no effect of intravenous steroids, but the patient's condition resolved after administration of Infliximab.

CONCLUSION

This case report supports the same treatment for gastritis as for colitis, which is in line with current guidelines.

Key words: Gastritis; Immune checkpoint inhibitors; Nivolumab; Case report; Immune-related adverse events; Infliximab

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Core tip: Lower gastrointestinal side effects, such as diarrhea and colitis, caused by immune checkpoint inhibitors are well described, but upper gastrointestinal side effects are less frequently reported. Here, we present a case of severe corticosteroid refractory

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gastritis induced by Nivolumab. The patient's symptoms resolved after administration of Infliximab. The treatment was in line with current guidelines for treatment of gastritis.

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) have shown to be effective drugs against various malignancies and are now standard of care for several types of advanced tumors. Current ICIs release some of the brakes in the immune system and thereby reinforce T-cell destruction of tumor cells by blocking regulatory cytotoxic T-cell lymphocyte-associated protein 4, programmed death receptor-1 (PD-1), or the PD-1 ligand^[1]. This may also lead to several immune-related adverse events (irAE), especially during Ipilimumab/Nivolumab double therapy^[2].

As the use of ICIs becomes more widespread, the knowledge of the irAEs of these drugs is increasingly important. The most common side effects are skin reactions and lower gastrointestinal (GI) side effects, and treatment of these reactions have been thoroughly described^[3,4]. However, only very few cases of patients with upper GI-tract immune mediated reactions have been reported in the literature and these have mainly been managed with prednisone monotherapy^[5-7].

Here we present a patient who developed a severe steroid refractory gastritis after Nivolumab monotherapy that required biological treatment with Infliximab.

CASE PRESENTATION

Chief complaints

A 16-year-old woman treated with adjuvant Nivolumab presented with vomiting, nausea, and weight loss.

History of present illness

The patient was diagnosed with stage IIIb malignant melanoma (T3bN1aM0). Both the primary melanoma and sentinel lymph node were radically removed. The patient was offered adjuvant treatment with Nivolumab (anti-PD-1), with 6 mg/kg administered every 4 wk^[8]. Initially, the patient tolerated the treatment well with only a small rise in plasma alanine transaminase compatible with a grade 1 hepatitis^[9]. An ultrasound examination of the liver was performed without any abnormalities observed.

After the sixth series of Nivolumab, the patient presented with anorexia, vomiting, nausea, upper abdominal pain, and a weight loss of approximately 3 kg (Table 1). The patient was admitted and received a short low-dose prednisone treatment for 4 d (40 mg methylprednisolone on the first day followed by 25 mg prednisone for 3 d) with a little initial symptomatic effect. The patient was discharged after 3 d, but readmitted 10 d later because of worsening of her symptoms with dehydration, vomiting, and stomach pain.

History of past illness

The patient had no comorbidities. There was no history of prior gastroenterological symptoms.

Personal and family history

The patient did not smoke or consume alcohol. There was no noteworthy family medical history.

Physical examination

Physical examination showed a pale and dehydrated patient with a weight loss of 3 kg. The abdomen was soft but revealed tenderness in the epigastrium.

Table 1 Timeline

Time	Event	Findings
June 2018	Removal of mole on the right thigh at the general practitioner	Pathology showed malignant melanoma, 2.1 mm. Level IV
August	Re-excision of malignant melanoma and sentinel node biopsy	Stage IIIb malignant melanoma. (No open protocol for adjuvant treatment)
September	PET-CT	No metastases
December	Multidisciplinary team-conference	Referred to the oncology department
January 2019	Started adjuvant Nivolumab treatment	
March	Nausea and stomach pain	Grade 1 hepatitis (ALT 129 U/L)
April	Ultrasound of liver because of elevated ALT	Normal
May	Decline in ALT (ALT 47 U/L), 6 th dose of Nivolumab	
June 21-24	First admission for 3 d with nausea, stomach pain, and vomiting; Cerebral MRI	Short prednisone treatment with initial effect. No brain metastasis
July 1	PET-CT (Figure 1)	FDG-uptake in the gastric wall
July 3	Second admission with vomiting, stomach pain, and nausea	ALT 85 U/L, albumin 23 g/L
July 4	EGD and EUS (Figure 2); Initiated methylprednisolone 80 mg iv.	Gastritis. Erythematous mucosa with severe, fibrinous erosions. Acute and chronic inflammation
July 10	First dose Infliximab	
July 11	Discharged; continued prednisone	
July 18	Initiated tapering of prednisone	
July 24	Second dose Infliximab	
August 8	EGD	Slight to moderate gastritis without ulcerations and fibrinous membranes. Improvement compared to the first EGD
September 17	PET-CT (Figure 4)	No FDG uptake in the gastric wall
September 26	Discontinued prednisone	

ALT: Alanine transaminase; PET-CT: Positron emission tomography with computed tomography; EGD: Esophagogastroduodenoscopy; EUS: Endoscopic ultrasound; MRI: Magnetic resonance imaging; FDG: Fluorodeoxyglucose.

Laboratory examination

Blood tests showed a slight elevation in alanine transaminase (91 U/L; reference range 10-45 U/L) compatible with grade 1 hepatitis. Additional blood tests, including thyrotropin and cortisol, were in normal range.

Imaging examination

At first admission after the sixth Nivolumab dose, a cerebral magnetic resonance imaging was performed to rule out metastases to the brain. In the following week a positron emission tomography with computed tomography (PET-CT) was performed. Abnormal fluorodeoxyglucose uptake was demonstrated in the gastric wall, especially around the corpus antrum (Figure 1). Linitis plastica was suspected and an esophagogastroduodenoscopy (EGD) with supplementary endoscopic ultrasound (EUS) was performed. The EGD showed a vulnerable mucosa with a white fibrine-like membrane in the antrum, corpus, and fundus. EUS demonstrated increased thickening of the gastric wall to 13 mm. No focal malignant lesions were suspected, and the finding was interpreted as inflammation. Macroscopically, the mucosa was erythematous with severe fibrinous erosions (Figure 2).

Pathological findings

The initial endoscopic examination was compatible with chronic active pangastritis (Figure 3A and B). Biopsies from fundus, corpus, and antrum ventriculi showed severe changes with ulceration, crustation, and only scattered glands. The glandular epithelium showed very reactive changes, apoptosis, neutrophilic inflammation, and crypt abscesses, as well as intraepithelial lymphocytosis (45 per 100 epithelial cells). The lamina propria showed a diffuse, full thickness lymphoplasmacytic inflammatory infiltrate. Epithelial granulomas, thickened subepithelial collagen layer, or prominent eosinophils, were not observed. There were no signs of malignancy, CMV infection, or *Helicobacter pylori*. Epstein Barr virus serology showed positive Epstein Barr virus



Figure 1 The first positron emission tomography with computed tomography after the patient presented with upper gastrointestinal symptoms. The scan showed abnormal fluorodeoxyglucose uptake in the gastric wall, especially around the corpus antrum.

nuclear antigen IgG corresponding with a previous infection.

FINAL DIAGNOSIS

The inflammation was interpreted as a severe immune related side effect to the Nivolumab treatment.

TREATMENT

Upon the second admission, the patient was treated daily with high-dose steroid (80 mg methylprednisolone) intravenously along with a proton pump inhibitor (PPI; 40 mg) with only minor relief of her symptoms. She still presented with vomiting and

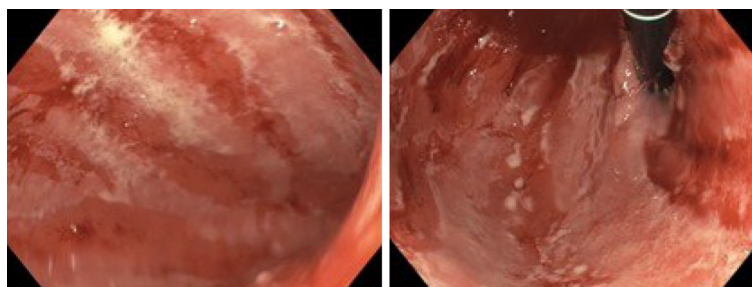


Figure 2 Esophagogastroduodenoscopy before treatment. The gastric wall was erythematous with severe fibrinous erosions of the mucosa.

nausea, and had trouble eating and drinking sufficiently. Albumin levels deteriorated to 23 g/L (reference range 37-48 g/L). Because of insufficient improvement on the intravenous methylprednisolone, the patient received Infliximab (5 mg/kg) 6 d after the initial steroid dose. She continued oral high dose steroid (100 mg prednisone) and PPI, which was briefly increased to 40 mg twice daily. Her symptoms improved temporarily for a week after receiving Infliximab, but because of continued nausea and light vomiting, she received a second dose of Infliximab 2 wk after receiving the first dose. Both PPI and prednisone were tapered during follow up.

OUTCOME AND FOLLOW-UP

A new EGD was performed 4 wk after the patient received the first dose of Infliximab. This showed improvement with slight to moderate gastritis, but without ulcerations and fibrinous membranes. In the antrum area, vulnerable mucosa was observed when touching with the endoscope. The EUS showed a stomach wall measuring 5-8 mm, but still 13 mm in the fundus. The findings were interpreted as improvement compared to the first EGD but still with some inflammatory changes remaining after the two Infliximab doses. Accordingly, the histology was without ulceration and regenerated mucosa (Figure 3C). The glandular epithelium showed mild to moderate chronic active activity. Acute inflammation with neutrophilic inflammation was seen in areas. There was still intraepithelial lymphocytosis (20 lymphocytes per 100 epithelial cells), but only few apoptotic cells were found. The lamina propria still showed increased lymphoplasmacytic inflammation, although less pronounced full thickness. There was no evidence of epithelial granulomas, thickened subepithelial collagen layer or prominent eosinophils. Likewise, there were no signs of malignancy or *Helicobacter pylori*.

The symptoms of the patient gradually improved under continuous tapering of steroids, which was discontinued after approximately 3 mo. There has been no need for a third dose of Infliximab to this point. The first status PET was performed 10 wk after the first Infliximab treatment and 3.5 mo after receiving the last Nivolumab dose and showed normalization with no fluorodeoxyglucose uptake in the gastric wall (Figure 4).

DISCUSSION

We present a case of severe corticosteroid refractory pangastritis, without Crohn-like pattern, in a young woman after receiving adjuvant Nivolumab for high-risk melanoma.

GI irAEs, such as colitis with diarrhea, are well known irAEs for all ICIs. Nivolumab monotherapy is less toxic than Ipilimumab alone or when used in combination therapy^[2,10]. The rate of grade 3/4 GI tract side effects caused by Nivolumab treatment alone is around 1%-2%^[2,3]. The incidence of diarrhea is reported to be 19%^[9]. Upper GI-tract toxicity such as nausea and vomiting, as presented in this case, are much less common^[10].

In guidelines, the recommended treatment for gastritis grade 2 or higher is similar to the management of colitis^[9,11] with corticosteroids at 1-2 mg/kg bodyweight. If there has been no sufficient effect within 3-5 d, biological treatments like Infliximab or Vedolizumab are recommended^[4,9].

Only a few case reports in the literature showed gastritis as a side effect to ICIs. Two of the reported cases had been treated with Nivolumab^[5,12], in one of the cases

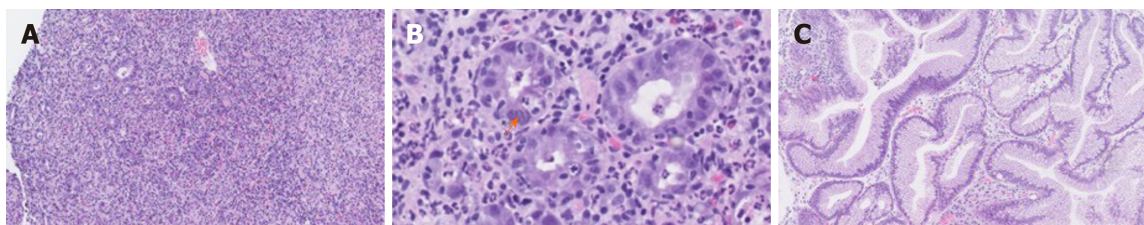


Figure 3 Imaging of histopathology. A, B: Diffuse chronic active pangastritis with ulceration and only scattered glands. Neutrophilic inflammation and crypt abscesses increased intraepithelial lymphocytes and apoptosis (arrow) (A: 100 ×; B: 400 ×); C: Regenerated epithelium with focal acute inflammation (100 ×).

symptoms first presented 6 mo after the treatment^[5]. Another was treated with Ipilimumab, but had previously been treated with Nivolumab^[6]. Others were treated with Pembrolizumab^[13], Ipilimumab monotherapy, or Ipilimumab and Nivolumab combination therapy^[7]. One case in Johncilla *et al*^[7] received Nivolumab monotherapy but developed only a discrete gastritis together with colitis. In one case, coexistence of *Helicobacter pylori* was observed^[6].

In Johncilla *et al*^[7], 8 of the 12 patients were treated with steroid monotherapy, 2 patients received Infliximab treatment and 2 patients were not in need of any treatment. Both the patients in need of Infliximab also had concurrent colitis. The paper did not highlight whether it was colitis or gastritis, which necessitated Infliximab therapy. In Boike *et al*^[5], Nishimura *et al*^[6] and Calugareanu *et al*^[12], the patients were treated with intravenous corticosteroids and PPI alone. In another study, no information was given as to whether corticosteroid was needed^[13].

Johncilla *et al*^[7], describes the histologic pattern of gastric irAEs and possible differential diagnosis. The most common pattern seen in the untreated form was a diffuse chronic active gastritis. Remaining patients showed a focal enhancing gastritis pattern similar to the changes seen in Crohn's disease. The two patients that received Infliximab therapy for resolution of their symptoms had both developed a Crohn's-like pattern. In our patient, we found ulceration and a severe diffuse chronic active pangastritis without evidence of granulomatous inflammation or focal enhancing gastritis, reminiscent of the histopathology seen in Crohn's disease. However, with such pronounced changes it may be difficult to distinguish between the two.

Even though upper GI tract symptoms are rarely reported during ICI treatment, signs of inflammation in the upper GI-tract might be present. A study on enterocolitis in 39 patients treated with anti-T-cell lymphocyte-associated protein 4 antibodies showed that 9 of the 22 patients, in which an EGD was performed, had coexistent gastritis. However, it was not reported if these patients showed any symptoms of gastritis^[11]. Similar results were found in another study on GI irAEs in 20 patients treated with an anti-PD-1 antibody^[14]. In this study 13 of the patients had an abnormal EGD. The main findings were mucosal erythema, but in two of the cases, the EGD showed necrotizing gastritis.

A recent retrospective single-center study^[15] investigated patients who developed upper GI symptoms in need for EGD within 6 mo after having received ICIs. This was only present in 60 out of 4716 cases, 23 of which required hospitalization. Fourteen patients were treated with Infliximab or Vedolizumab, but only one of these patients had isolated upper GI tract involvement. The remainder had concurrent lower GI tract involvement.

In this present case report the patient was treated for severe gastritis according to the guidelines for colitis with initially corticosteroids intravenously and afterwards Infliximab because of insufficient effect of the corticosteroids alone. On this treatment, the patient's clinical symptoms resolved completely and on PET-CT within three and a half months after the last Nivolumab dose.

CONCLUSION

Severe gastritis, as presented in this case, is a much rarer adverse event for ICIs, especially Nivolumab monotherapy, than lower GI symptoms like colitis. However, the knowledge and awareness of this complication is important in all combinations of ICIs. Patients with severe ICI induced gastritis deteriorates very fast due to insufficient nutrition. The usage of ICIs expands and in order to give proper treatment for immune mediated gastritis in time, further studies of the histopathology and response to treatment are required. No controlled clinical studies have been published on the management of upper GI tract symptoms. However, current guidelines



Figure 4 The second positron emission tomography with computed tomography performed 10 wk after the first Infliximab administration. This showed a normal gastric wall with no fluorodeoxyglucose uptake.

recommend timely biological treatment as for ICI induced colitis. The case report supports this recommendation.

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Efficacy of bevacizumab-containing chemotherapy in metastatic colorectal cancer and CXCL5 expression: Six case reports

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Abstract

BACKGROUND

In metastatic colorectal cancer (mCRC), the anti-vascular endothelial growth factor drug bevacizumab (BVZ) plus chemotherapy significantly improves progression-free survival compared to chemotherapy (CT) alone. This benefit is not, however, observed in all patients. While increased chemokine CXCL5 gene expression promoting angiogenesis has been proposed as a prognostic mCRC biomarker, few studies have examined its relationship with drug efficacy. This study sought to analyze tumor CXCL5 gene expression in six patients with different efficacy of BVZ-containing CT in terms of the tumor response to treatment.

CASE SUMMARY

We report six cases of stage IV KRAS-mutated mCRC. Patients were given first line treatment with BVZ-containing chemotherapy in University Hospital of Fuenlabrada. The six patients differed in terms of primary tumor location (right/left side), tumor burden (mostly hepatic and peritoneal disease) and clinical disease course. Before treatment onset, total RNA was isolated from paraffinated tumor biopsy specimens and CXCL5 gene expression quantified

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Informed consent statement:

Informed written consent was obtained from the patients for publication of this report.

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through conventional RT-qPCR procedures. Our main finding was that CXCL5 expression levels were several times higher in three patients with lower progression free survival (under 6 mo) from the start of treatment.

CONCLUSION

A higher expression of CXCL5 was observed in the three patients showing worse tumor response to treatment.

Key words: Colorectal cancer; Bevacizumab; Chemokine CXCL5; Gene expression; Progression-free survival; Case report

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Core tip: Although compared to chemotherapy (CT) alone, bevacizumab-containing CT leads to a significantly better tumor response in metastatic colorectal cancer patients, many do not benefit from this regimen probably due to resistance mechanisms or readjustment of proangiogenic pathways. While CXCL5 expression has been described to predict a poor prognosis in different cancers, its relationship with the efficacy of CT regimens has been scarcely addressed. Our three patients showing CXCL5 higher expression (6 times the levels recorded in the others) showed a poor response in terms of progression-free survival. Our observations provide direction for future studies designed to examine in metastatic colorectal cancer patients treated with bevacizumab-containing therapy, the possible association between CXCL5 gene overexpression and a poor response to treatment with angiogenic drugs.

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INTRODUCTION

Angiogenesis is essential for tumor growth. It has been established that the vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play major roles in angiogenesis associated with advanced cancer. The monoclonal antibody bevacizumab (BVZ), an anti-VEGF drug directed against the vascular endothelium, is a common component of combination chemotherapy (CT) regimens used in patients with metastatic colorectal cancer (mCRC). Several authors have reported significant improvements in progression-free survival (PFS), overall survival (OS) and response rate in mCRC patients compared to CT alone^[1,2]. However, in a significant number of patients there is no meaningful benefit probably because of the acquisition of resistance mechanisms involving activation of compensatory proangiogenic pathways or tumor recruitment of cells that produce proangiogenic factors^[3].

CXCL5 is a chemokine that binds the G-protein-coupled receptor chemokine receptor 2 (CXCR2) to recruit neutrophils, promote angiogenesis and remodel connective tissues, playing a role in cancer cell proliferation, migration, and invasion^[4,5]. While several studies during the past decade have examined the use of CXCL5 gene expression as a biomarker for cancer diagnosis or prognosis, *e.g.*, Wu *et al*^[6], few investigations have explored the relationship between CXCL5 expression and treatment efficacy. In this paper, we describe six patients with mCRC who showed a different response to BVZ-containing CT, and discuss the possibility of a relationship between differential CXCL5 tumoral gene expression and the efficacy of the regimen used in terms of PFS.

CASE PRESENTATION

Chief complaints

We identified six Caucasian patients with metastatic colorectal cancer. There were 3 men and 3 women with a median age of 70 years at diagnosis (range: 47-81 years). Patient characteristics are summarized in Table 1. The patients were referred to department of oncology for clinical and therapeutic evaluation.

History of present illness

Each case was diagnosed due to slightly different symptoms, some of them related:

Case 1: This was a 62-year-old woman who was studied from aggravated constipation, tenesmus, abdominal pain and rectal bleeding in November 2012 with stage IV KRAS-mutated rectal cancer with a low disease burden (lung and liver metastasis).

Case 2: This patient was a 47-year-old presented at the emergency room with acute abdominal pain less than 48 h in right iliac pit in September 2016. She suffered bowel obstruction and required left colectomy, adnexectomy and cytoreduction. She was diagnosed with stage IV (metastases in peritoneum, ovary, liver) KRAS-mutated sigmoid cancer.

Case 3: A 71-year-old man was studied due to weight loss and occasional vomiting for few months in December 2015. He was diagnosed with stage IV (liver metastases), KRAS-mutated, right colon cancer.

Case 4: A 62-year-old man, who was diagnosed with a right KRAS-mutated colon tumor with lung metastasis in May 2011. He presented with diarrhea and weight loss for 6 months.

Case 5: This case was an 81-year-old man with stage IV, KRAS-mutated sigmoid cancer (lung metastasis) who underwent primary tumor resection in February 2014. He debuted with rectal bleeding for few weeks before without pain or weight loss.

Case 6: The final case was that of a 75-year-old woman who was diagnosed in March 2015 with mucinous appendix KRAS-mutant colon cancer. She was admitted due to right iliac pit pain and increased abdominal size for two months.

History of past illness

Case 1: Her personal background consisted of high blood pressure, hypercholesterolemia and osteoporosis.

Case 2: She presented severe sleep apnea-hypopnea syndrome, multinodular goiter and uterine myomas.

Case 3: He had high blood pressure and hypercholesterolemia under treatment with optimal control.

Case 4: His clinical record was only based on pulmonary tuberculosis and nodular hyperplasia of thyroid.

Case 5: He presented just hypercholesterolemia and chronic lumbalgia.

Case 6: Unremarkable.

Personal and family history

Case 1: She had one brother with gastrointestinal stromal tumor and father with lung cancer and smoking story.

Case 2: Her father had sigmoid cancer and her mother suffered of breast cancer.

Case 3 and 4: Unremarkable.

Case 5: Deceased father with colon cancer at 70 years old and one living brother with previous rectal cancer diagnosed with 80 years-old.

Case 6: Unremarkable.

Physical examination upon admission

Case 1: Her vitals were normal as well as cardiopulmonary auscultation. She had only lower abdominal pain at hypogastric region.

Table 1 Patient characteristics

Characteristics	Data (n = 6)
Gender	
Male	3 (50)
Female	3 (50)
Age (yr)	70 (47-81)
Tumor stage IV ¹	6 (100)
Localization ¹	
Right	2 (33.3)
Sigmoid	2 (33.3)
Rectal	1 (16.7)
Mucinous appendix	1 (16.7)
KRAS status ¹	
Mutated	6 (100)
Normal	0 (0)

¹Evaluated before chemotherapy. Data are n (%).

Case 2: She presented pain in the upper abdominal quadrant and associated peritonism.

Case 3: He presented with abdominal pain without peritonism.

Case 4: Vitals were normal, no abdominal pain, masses palpation or peritonism.

Case 5: He just presented discrete abdominal pain, without any other signs.

Case 6: She presented with shortness of breath due to mechanical impingement on the diaphragm. Besides physical examination of the abdomen presented bulging of the flanks and shifting dullness, and edema in lower legs.

Laboratory examinations

Case 1: Blood test without abnormalities except from high level of carcinoembryonic antigen (CEA) 29.7 ng/mL (0.5-5).

Case 2: She presented high levels of serum markers CEA 83.7 ng/mL and carbohydrate antigen (CA19.9) 84 UI/mL (0-35) and anemia hemoglobin 10.5 g/dL.

Case 3: Blood test with anemia 11 g/dL and high CEA 69.7 ng/mL.

Case 4: His blood test presented hemoglobin 12.5 g/dL with low ferritin and normal serum markers.

Case 5: Blood test was normal without anemia or high serum markers.

Case 6: Hemoglobin 11.8 g/dL and serum markers CEA 930 ng/mL and CA 19.9 75 UI/mL.

Case 1-6: Before treatment onset, total RNA purification was performed on paraffinated tumor tissue biopsy specimens using Promega kits and Maxwell techniques from three five μ m-slices. CXCL5 gene expression was quantified through conventional RT-qPCR techniques (Biorad). The housekeeping gene *G3PDH* was used to normalize CXCL5 gene expression. Table 2 displays normalized gene expression for CXCL5 gene from each biopsies and PFS after BVZ-containing treatment onset. The three patients showing CXCL5 higher expression (6 times the levels recorded in the others) showed a poor response in terms of PFS (Table 2).

Imaging examination

Case 1: Computed tomography was performed at the beginning of diagnosis with rectal tumor in radiological stage T3N1M1 with multiple hypodensive lesions, in both lobes and pulmonary nodules.

Case 2: Computed tomography showed sigma neoplasm with hepatic metastatic disease, apart from multiple local adenopathies. Besides, complex cystic mass in right ovarian of 13 cm \times 10 cm was presented that could be compatible with ovarian

Table 2 Normalized CXCL5 gene expression levels and progression-free survival after bevacizumab-containing treatment onset in the six cases reported in this study

Case number	1	2	3	4	5	6
PFS after BVZ-containing treatment onset (mo)	18	12	12	3	6	3
Overall PFS after BVZ-containing treatment onset (mo)	12 (12-18)			3 (3-6)		
Normalized CXCL5 gene expression	10.700	3.655	0.002	23.430	20.390	32.900
Overall normalized CXCL5 gene expression	3.655 (0.002-10.700)			23.430 (20.390-32.900)		

Overall data are median (range). BVZ: Bevacizumab; PFS: Progression-free survival.

metastases.

Case 3: Colonoscopy showed mass in right colon with stenotic diameter. Besides computed tomography was performed to confirm colon cancer stage T4a N1 M1 with regional adenopathies, peritoneal implants with ascites and three metastatic liver lesions.

Case 4: Computed tomography presented right colon tumor in radiological stage T4 N1 M1 with regional adenopathies and two nodules in right lung lobe.

Case 5: Colonoscopy showed neoformation that occupied more than 90% up to 30 cm of anal margin where the light did not allow the endoscope to pass. Computed tomography scan confirmed sigma stenosis tumor with radiological stage T3N2M1 due to lung nodules.

Case 6: Computed tomography scan showed signs of extensive pseudomyxoma and inframesolic secondary to mucinous colon carcinoma with abundant ascites and peritoneal implants and small bowel infiltration.

FINAL DIAGNOSIS

The final diagnosis of the six presented cases was stage IV KRAS-mutated colorectal cancer.

TREATMENT

Case 1

Treatment was 5-fluorouracil and irinotecan (FOLFIRI) plus BVZ for 8 cycles, to which she showed a partial response, short-course preoperative radiotherapy, and two surgeries (first for the primary tumor, interval CT for two cycles without an antiangiogenic molecule and then left hepatectomy and radiofrequency ablation in the right liver lobe). She was maintained on the same CT regimen until lung progression detected 18 mo after diagnosis, and therapy was switched to FOLFOX (oxaliplatin, 5-fluorouracil, leucovorin). The response was rapid disease progression. For one year, she received third line treatment with FOLFIRI plus aflibercept until bone metastasis in October 2014. She then underwent radiation therapy and was started on fourth line regorafenib.

Case 2

She started on FOLFOX plus BVZ for 8 cycles with a partial response. A right hepatectomy and ALPSS procedure (Associating Liver Partition and Portal vein Ligation for Staged hepatectomy) were performed in May 2017 requiring long hospitalization. In total, she received 13 CT cycles. In October 2017, she was readmitted because of peritonitis but two months later there was disease progression. This patient received second line treatment with FOLFIRI-aflibercept for six months to which she responded.

Case 3

Treatment was FOLFOX plus BVZ for 3 cycles plus advanced surgery because of bowel blockage. The patient received 12 doses of this BVZ-containing CT and there was no evidence of disease during 12 mo. In March 2017 he was treated with radiofrequency ablation and FOLFIRI-aflibercept CT because of hepatic relapse, to

which he responded; in December 2018 he underwent cytoreductive surgery.

Case 4

He was first given 4 cycles of FOLFOX without bevacizumab due to a risk of bowel perforation. Three months later, the disease progressed and he underwent surgery for the primary colon tumor because it was symptomatic. Following three months of further CT based on FOLFIRI plus BVZ with no response, he was switched back to FOLFOX with the same anti-VEGFR for 8 cycles more.

Case 5

He completed adjuvant treatment with capecitabine for only 3 mo due to cardiac ischemia. In August 2014, there was lung and lymph disease progression, so he was started on capecitabine plus BVZ for 12 cycles.

Case 6

She was treated with hyperthermic intraperitoneal CT surgery and first-line CT based on FOLFOX plus BVZ for 13 cycles resulting in disease stabilization. This was followed by treatment with 5-fluorouracil plus BVZ due to neurotoxicity and myelotoxicity until progression in December 2015. She continued with FOLFIRI plus BVZ with progression observed after 3 mo and several hospitalizations required due to acute pain. Finally, the patient received third line TAS102 (trifluridine/tipiracile).

OUTCOME AND FOLLOW-UP

Case 1

In January 2016 she was admitted to hospital due to acute exacerbation of chronic pain, clinical worsening and died at 66 years.

Case 2

She was hospitalized due to liver failure (in February 2018) and died.

Case 3

Currently he is well and there is no evidence of disease at 74 years-old.

Case 4

He did not show improvement and finally died in May 2012.

Case 5

The treatment capecitabine plus BVZ for 12 cycles was ineffective and lung involvement progressed until death in August 2016 at 84 years-old.

Case 6

The response was rapid tumor progression until her death in July 2017.

DISCUSSION

Colorectal cancer is the most common gastrointestinal tract cancer and is associated with a high morbidity and mortality in both men and women^[7]. Both its early detection and the search of targeted therapies are critical for improving outcomes and reducing CRC patient mortality. Biomarkers for diagnosis, prognosis and targets of therapy are urgently needed to improve survival rates. According to current guidelines, a first-line treatment option for mCRC is the combination of BVZ plus CT consisting of 5-fluorouracil and oxaliplatin or irinotecan, especially in patients with the KRAS-mutated form of disease^[8]. However, there is no general consensus as to whether patient selection for this treatment should be based only on KRAS status or whether other clinical characteristics (primary tumor site, histologic subtype, *etc.*) should be also taken into account^[9]. Further factors that should also be considered are molecular markers of CRC as they could play a determining role in tumor progression. Unfortunately, these have not yet been identified.

Although BVZ antiangiogenic therapy is considered a good treatment option for mCRC, there is still scarce knowledge regarding its efficacy and resistance acquisition among patients. Reports exist in the literature on the benefits of BVZ-containing CT over CT alone in terms of significantly better PFS and OS detected in mCRC patients^[2,10]. However, only 61% of mCRC patients treated with BVZ-containing CT show an objective response (complete or partial)^[10]. It remains to be determined why some tumors prove resistant to BVZ either from the start of treatment or after several

months.

Chemokines produced by tumor and stromal cells are involved in the distribution of tumor-associated leukocytes, metastasis, angiogenesis and tumor growth^[11]. The chemokine CXCL5, also called epithelial neutrophil-activating peptide 78-ENA-78-, binds the G-protein CXCR2 to recruit neutrophils and to promote angiogenesis, playing a role in cancer cell proliferation, migration, and invasion^[12]. Because of the important role played by these molecules in cancer processes, several chemokines have been measured in different types of tumors, and abnormal expression levels observed in many of them (*e.g.*, Samarendra *et al.*^[13]). The elevated expression of CXCL5 has been associated not only with gastric cancer, prostate cancer, endometrial cancer, squamous cell cancer, hepatocellular carcinoma and pancreatic cancer, but also with advanced tumor stages and with metastatic potential^[14]. Several studies addressing the use of differential CXCL5 gene expression as a biomarker for cancer diagnosis or prognosis have been published in the past decade, *e.g.*, Wu *et al.*^[6]. However, the relationship between CXCL5 expression and drug efficacy has been scarcely investigated. Sunitinib is a multitarget tyrosine kinase inhibitor approved for the treatment of renal cell carcinoma. In this setting, plasma CXCL5 levels have been associated with therapy efficacy, but no correlation was found with BVZ-containing CT by Giuliano *et al.*^[15]. The authors Li *et al.*^[16] reported the down-regulation of another chemokine, CXCL1, in carcinoma-associated fibroblasts isolated from breast tumors as possibly responsible partially for the efficacy of letrozole, a non-steroidal aromatase inhibitor.

Germline genetic variability within genes related to the angiogenesis pathway could be associated with differences in resistance to anti-angiogenesis therapy among patients. de Haas *et al.*^[17] studied SNP variants of VEGF-C, EPAS1 and CXCR2 genes in blood samples from patients treated with BVZ-containing CT. These authors identified the CXCR2 variant (rs2234671) as predictive of bevacizumab treatment outcome in terms of PFS. Wild-type CC carriers were characterized by prolonged PFS in different types of tumors such as colorectal, pancreatic, lung, renal, breast, and gastric. Gerger *et al.*^[18] obtained similar results in a cohort of mCRC patients treated with BVZ and oxaliplatin-based CT.

Chen *et al.*^[19] recently proposed that CXCL5 is an important angiogenic factor that can promote cell metastasis through tumor angiogenesis in CRC. This research group used recombinant human CXCL5 in *in vitro* experiments and observed enhancement of their tube formation ability, proliferation, and migration *via* activation of the AKT/NF- κ B/FOXO1/VEGF-A pathway in a manner that was very dependent on CXCR2, the receptor of CXCL5. Besides, in *in vitro* studies, it was found that silencing of CXCR2 or these pathways could attenuate tube formation ability, proliferation, and migration. Similarly, increased CXCL5 expression was noted to augment microvessel density in an *in vivo* mouse tumor model. Thus, blocking overexpression of the CXCL5/CXCR2 axis could be a promising treatment strategy for CRC patients. These authors established a possible relationship between the CXCL5/CXCR2 pathway and VEGF-A expression. It is known that high plasma/intratumoral VEGF-A levels at baseline are related to a poor treatment response (reduced PFS and OS) to BVZ-based chemotherapy in mCRC^[20].

CXCL5 could be considered a significant predictor of tumorigenesis and progression because of the relationship observed between its overexpression and enhanced angiogenic pathway activity. Although CXCL5 expression has been described as a predictor of a poor prognosis in different types of cancer (prostate, endometrial, hepatocellular, and pancreatic)^[21], the current lack of data determines a need to explore the possibility of a relationship between CXCL5 expression and anti-angiogenic drug efficacy. Our three patients showing CXCL5 higher expression showed a poor response in terms of PFS (Table 2). The number of cases described here is a clear limitation for statistical analysis, but we consider this result a starting point for larger-scale studies. The hypothesis that an anti-VEGF drug such as BVZ may not counteract the overstimulation of microvessels and the search of better strategies as, for instance, the possibility of inhibition of the CXCL5/CXCR2 pathway in CXCL5 overexpressed patients could be a very interesting point of view in future enlarged studies.

CONCLUSION

Although it is known that CXCL5 is a vital angiogenic factor in different types of cancer, very little information is available regarding the effects of CXCL5 in angiogenesis related to CRC. In this study, six biopsies from patients with mCRC treated with BVZ-containing therapy were analyzed to quantify CXCL5 gene expression. Our main

finding was the higher expression of CXCL5 observed in the three cases showing the worst PFS. Our observations provide a starting point for future studies designed to examine the possible association between CXCL5 gene overexpression and a poor response to treatment with angiogenic drugs in mCRC patients.

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