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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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REVIEW

Gastrointestinal stromal tumors: A multidisciplinary challenge

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors located in the alimentary tract. Its usual manifestation is gastrointestinal bleeding. However, small asymptomatic lesions are frequently detected as incidental finding. Characteristically, most GISTs (> 95%) are positive for the KIT protein (CD117) by IHC staining and approximately 80%-90% of GISTs carry a mutation in the c-KIT or PDGFRA genes. Mutational analysis should be performed when planning adjuvant and neoadjuvant therapy, due to its possible resistance to conventional treatment. The arise of tyrosine kinase inhibitor has supposed a revolution in GISTs treatment being useful as adjuvant, neoadjuvant or recurrence disease treatment. That is why a multidisciplinary approach to this disease is required. The correct characterization of the tumor at diagnosis (the diagnosis of recurrences and the evaluation of the response to treatment with tyrosine kinase inhibitors) is fundamental for facing these tumors and requires specialized Endoscopist, Radiologists and Nuclear Medicine Physician. Surgery is the only potentially curative treatment for suspected resectable GIST. In the case of high risk GISTs, surgery plus adjuvant

Imatinib-Mesylate for 3 years is the standard treatment. Neoadjuvant imatinib-mesylate should be considered to shrink the tumor in case of locally advanced primary or recurrence disease, unresectable or potentially resectable metastasic tumors, and potentially resectable disease in complex anatomic locations to decrease the related morbidity. In the case of Metastatic GIST under Neoadjuvant treatment, when there are complete response, stable disease or limited disease progression, complete cytoreductive surgery could be a therapeutic option if feasible.

Key words: Gastrointestinal stromal tumors; Surgery; Oncology; Radiology; Endoscopy; Nuclear medicine; Pathology; Disease management; Tyrosine kinase inhibitors; Gastroenterology

Core tip: The treatment of gastrointestinal stromal tumors with tyrosine kinase inhibitors represents the paradigm of the new era of molecular targeted therapy against cancer. During the last years, there have been improvements in the control of this disease and in the prognosis of these patients, deriving in hopeful perspectives in the management of these tumors partly thanks to the numerous specialists. In this work, we define the role of each specialist in the different clinical scenarios.

Sanchez-Hidalgo JM, Duran-Martinez M, Molero-Payan R, Rufian-Peña S, Arjona-Sanchez A, Casado-Adam A, Cosano-Alvarez A, Briceño-Delgado J. Gastrointestinal stromal tumors: A multidisciplinary challenge. *World J Gastroenterol* 2018; 24(18): 1925-1941 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1925.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1925

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumor located in the gastrointestinal (GI) tract. Most studies have reported the incidence of clinically relevant GIST between 10 and 15 cases per million; however, it is common to detect small asymptomatic lesions as incidental findings during abdominal surgery or in radiological or endoscopic studies, so GIST cases are often misdiagnosed[1,2].

The majority of studies have reported an increase in incidence since 2000; nevertheless, this may be a consequence of improvements in diagnostic criteria rather than a true increase in incidence[3].

GISTs are more often located in the stomach (56%) followed by small bowel (32%), colorectum (6%), and esophagus (< 1%). Sporadically, it may affect the omentum, mesentery, and peritoneum[4]. Liver and peritoneum are the most common locations for distant metastases where they appear up to 47% at the time of diagnosis[5]. Pulmonary metastases, which are highly frequent in soft tissue sarcomas, are uncommon in the case of GISTs.

Interstitial cells of Cajal (ICCs) are recognized as the precursor cells of GISTs being implicated in the regulation of gut peristalsis. They are considered the pacemaker cells of the gastrointestinal tract and are immunostained by antibodies against CD117 (KIT) like GISTs[6,7]. ICCs are located between the layers of the muscularis propria in the interface between the autonomic innervation of the gastrointestinal wall and the smooth muscle, having immunophenotypic and ultrastructural features of smooth muscle and neuronal differentiation[8].

Characteristically, most GISTs (> 95%) are positive for KIT (CD117) protein staining. Approximately 80%-90% of GISTs carry a mutation in the *c-KIT* gene (80%) or platelet-derived growth factor receptor alpha (PDGFRA) gene, which code for type III receptor tyrosine kinases[9].

Traditionally, GIST tumors have been characterized by their resistance to conventional chemotherapy and radiotherapy treatments. Nevertheless, in 2002, the appearance of the tyrosine kinase inhibitor, Imatinib-Mesylate, was the first to be used to treat metastatic disease and currently has been introduced as an adjuvant or neoadjuvant. This drug was suggested to revolutionize treatment of these tumors that normally requires a multidisciplinary approach, which involves numerous specialists such as physicians, endoscopist, surgeons, radiologists, oncologists, nuclear medicine physicians, or pathologists[10].

ROLE OF CLINICIAN

A high proportion of GISTs are asymptomatic, and frequently, they are discovered incidentally during an endoscopic study (It is common to notice the presence of a sub epithelial mass) or on radiological images obtained for another purpose. Incidental finding can cause a significant diagnostic delay. Currently, a significant number of patients presents with metastases at the time of diagnosis (up to 50% in some series)[5]. It is essential that physicians include GISTs in differential diagnosis due to the importance of early diagnosis in these cases.

Clinical manifestations depend on the location of the primary tumor. There is no difference between gender and mean age reported is approximately 60-70 years old[1,11].

Usually, these tumors are associated with nonspecific symptoms (early satiety, swelling) unless they ulcerate, bleed or grow enough to cause pain, obstruct, or present other manifestations related to their disproportionate size[12,13]. In the case of esophageal GIST, dysphagia represents the first specific symptom in this location[14].

In general terms, the most frequent manifestation is gastrointestinal bleeding, either evident or hidden, which may be associated with anemia and sometimes melena or hematemesis[15]; bleeding is the most frequent symptom in case of small intestine GISTs and often require urgent surgical intervention[16]. Because their silent growth tumors may be particularly large causing abdominal distention or a palpable mass and sometimes provoking intestinal obstruction (25%-40%); however, intestinal perforation has rarely been described[16,17].

Paraneoplastic syndromes are unusual in case of GISTs; however, some have been reported as consumptive hypothyroidism or hypoglycemia secondary to IGF-II production, so they should be included in the differential diagnosis when endocrine-metabolic symptoms appear[18,19].

Patients with multifocal disease are generally classified as advanced (metastatic) stage, but it should be taken into account, particularly in those cases with hereditary conditions, that multiple primaries may be possible[20].

In adults, GIST tumors have been associated with multiples syndromes as neurofibromatosis type 1 (NF1), Carney Triad syndrome and Carney Stratakis syndrome; GISTs associated with NF1 usually appear in the gastrointestinal tract and are usually multicentric. In these tumors, the KIT mutation is not characteristic, and they are usually positive for the CD117 antigen[21]; on the other hand, Carney's triad consists of epithelioid GISTs is associated with extra-adrenal paraganglioma and pulmonary chondroma. It lacks the conventional KIT and PDGFRA gene mutations and tends to present an indolent course[22]; the Carney Stratakis syndrome is extremely rare and is similar to Carney's Triad syndrome but lacks pulmonary chondroma and follows a benign course. Mutations have been identified in KIT or PDGFRA. The tumors are generally small, lack mitotic activity and arise in interstitial cells of Cajal[23].

Pediatric GISTs are assumed to be 1%-2% of all GISTs. Two subgroups exist: (1) with mutations (KIT or PDGFRA) or (2) without mutations (the most frequent). The patients are almost exclusively young women who develop gastric epithelioid GISTs, which are KIT types. Unlike adult GISTs, these tumors can spread to lymph nodes[24,25].

ROLE OF RADIOLOGIST

Computed tomography (CT) is the gold standard for imaging that is used to characterize any abdominal mass in addition to assessing its extent and the presence/absence of disease at a distance (GIST metastasize more frequently to the liver, omentum, and peritoneal cavity). Therefore, with suspicion of a tumor in the digestive tract, as in the case in question, an initial CT scan should be done. It should be noted that for the optimal performance of CT, oral and intravenous contrast should be administered in order to define the intestinal margins[26,27] (See Figure 1 and 2).

Magnetic resonance imaging (MRI) has a diagnostic performance comparable to CT and the advantage of lacking ionizing radiation; however, CT is the preferred initial imaging study for screening and staging of the disease. There are exceptions to this process; for example, there are patients who cannot receive intravenous contrast for various reasons (allergies, IR). In addition, MRI can sometimes be the choice for GISTs found in specific locations (such as the rectum) and is especially useful for evaluating the anatomical degree of surgery or for evaluating the suspicion of liver metastases[26] (See Figure 3).

The usual characteristic images seen on these images include the presence of a solid mass with a soft contour that is enhanced with intravenous contrast in the case of CT[28]. Very large tumors may appear more complex due to necrosis, haemorrhage, or degenerative components, and it may be difficult to identify the origin of a large mass due to exophytic growth[27].

With regard to the evaluation of response to treatment, patients are routinely subject to CT, and two-dimensional measurements are used to determine response, stability, or progression.

During neoadjuvant and adjuvant treatment, radiologists are mainly involved in the evaluation of the response to treatment with tyrosine kinase inhibitors (TKI). On the one hand, Response Criteria in Solid Tumors (RECIST) is the standard method used to measure the way in which a cancer patient responds to treatment. In order to apply the RECIST criteria, it is necessary to first identify representative and reproducible target lesions during follow-up; it should be taken into account that its great variability (fragmentation, poor definition, imaging technique, appreciation) in addition to the difficulty in measuring them, as occurs in mobile organs, cause intra-observer and inter-observer discrepancies. Assessments of the response will be made with the same technique used in the initial study, stating the duration of the response. The sum of the target lesions in the baseline study can be used to objectively monitor and assess the response. When a target lesion fragments during treatment, its parts will be measured, added up, and considered as a single lesion. The RECIST criteria are a series of published rules to establish the response to treatments and indicate when cancer patients improve ("respond"), stay the same ("stable"), or get worse ("disease progression[29,30].

In the other hand, the Choi Criteria[31] are useful in the evaluation of imatinib treatment of GISTs. In this case, the most characteristic feature is a decrease in the density of lesions associated with myxoid degeneration, hemorrhage, or necrosis. These criteria, based on CT studies, include tumor size, its density, and the appearance of intratumoral hypervascular nodules. They present a high correlation between the results obtained in CT and positron emission tomography (PET). CT

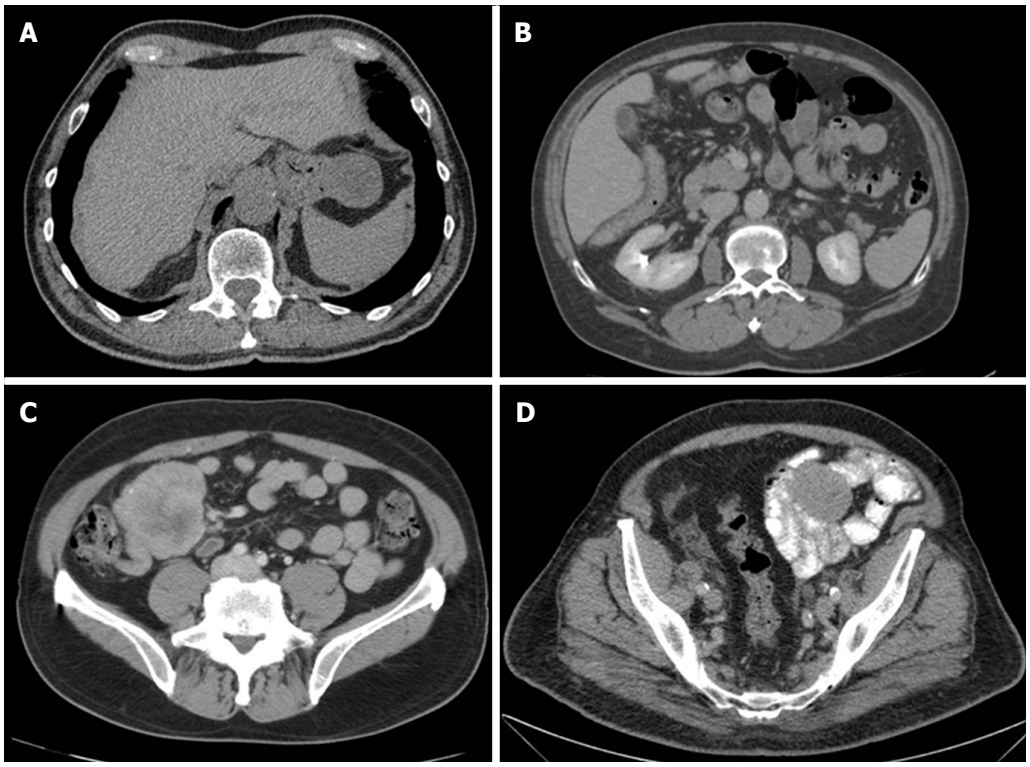

Figure 1 Localized gastrointestinal stromal tumors on computed tomography scan. A: Gastric; B: Duodenal; C: Ileal; D: Jejunal. A and B show respectively a gastric tumor and a duodenal tumor of exophytic growth with well-defined borders. Appreciate in C the different densities inside the tumor due to due to necrosis, haemorrhage, or degenerative components. D shows a jejunal GIST in left iliac fossa.

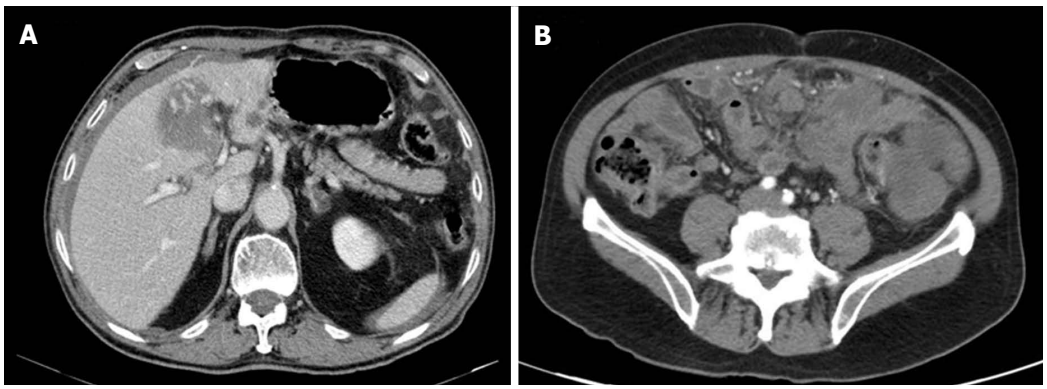

Figure 2 Metastatic gastrointestinal stromal tumors on computed tomography scan. A: Liver metastasis; B: Peritoneal metastasis "Gistosis". In A, it is appreciated a large hepatic metastasis in segment IV. B shows the CT of a patient with disseminated peritoneal disease "GISTosis".

should be performed in arterial phases (to see changes in vascularization and uptake) and portal (to measure tumor density)[32].

Role of interventional radiologist

As described previously, GISTs usually manifest as gastrointestinal bleeding. Transcatheter arterial embolization has proven to be a safe option for controlling gastrointestinal bleeding, thus preventing emergency surgery that would probably allow a more accurate diagnosis to be made and the best possible surgical plan to be executed[33].

Traditionally, preoperative percutaneous biopsy has not been used due to the possibility of tumor rupture or peritoneal spread of disease[8]. There are data supporting the finding that it may not increase the risk for GIST recurrence in those patients who receive adjuvant Imatinib after the biopsy was obtained. Percutaneous biopsy should be considered when it is necessary to plan preoperative treatment with TKI and endoscopic biopsy is not feasible[17,34].

In the case of patients with unresectable liver metastases, some local interventional modalities, such as transarterial embolization or radiofrequency ablation may be used; however, further studies are necessary to evaluate its effectiveness as adjuvant therapy or

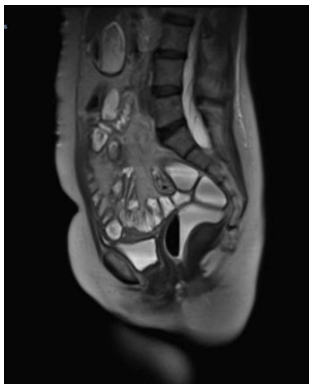

Figure 3 Rectal gastrointestinal stromal tumor on magnetic resonance.

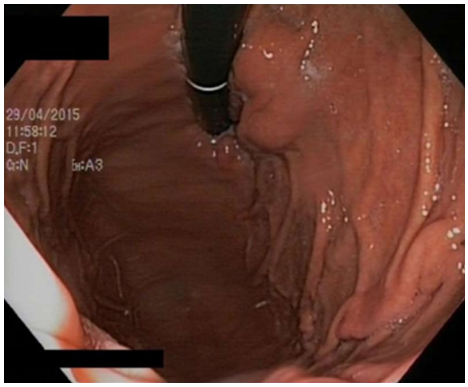

Figure 4 Characteristic endoscopic image of gastric gastrointestinal stromal tumor.

combined with TKI[35-37].

ROLE OF ENDOSCOPIST

In the presence of a gastric mass, endoscopy is indicated to characterize the lesion. GISTs and leiomyomas may appear as a submucosal mass with smooth margins and a normal overlying mucosa that protrudes in the gastric lumen, but sometimes, a central ulceration may be observed[38] (See Figure 4).

Simple endoscopy lacks the ability to accurately distinguish between intramural and extramural tumors. In this sense, Endoscopic Ultrasonography (EUS) has proved to be a valuable technique, being able to characterize such masses by identifying the layer of origin and allowing for acquisition of tissue by a guided puncture for anatomopathological diagnostic studies, which is suitable for immunohistochemical tests[39,40]. Standard endoscopic biopsies generally do not obtain enough tissue for a definitive diagnosis, and loop biopsies can cause a perforation and should generally be avoided[34].

Most GISTs originate within the muscularis propria although small lesions may originate in the muscularis mucosae. By EUS, GISTs are typically hypoechoic and present as homogeneous lesions with well-defined margins although there are a small number of described tumor cases of tumors that may have irregular margins and ulcerations (See Figure 5). Ultrasound (US)-guided biopsy forceps may also not obtain enough tissue, but its main utility is to exclude other lesions arising from the submucosa[39].

Preoperative biopsy is not generally recommended for a resectable lesion if there is high clinical and radiological suspicion of GIST, and the lesion is completely resectable. However, a preoperative biopsy is preferred to confirm the diagnosis if metastatic disease is suspected, if the neoadjuvant Imatinib is considered, or in cases in which there exists high operative morbidity or the diagnosis is not clear.

If a preoperative biopsy is performed, an US-guided biopsy is preferred to a percutaneous biopsy because of the risk of tumor capsule rupture and consequent peritoneal dissemination[34].

The combined use of cytological analysis and immunohistochemistry for KIT protein detection and expression and polymerase chain reaction (PCR) to detect KIT mutations allow the diagnosis of most of these lesions obtained by EUS - fine- needle aspiration Biopsy (FNAB). In a study of 65 patients undergoing EUS-FNAB for a submucosal lesion of the upper GI tract, among the 28 lesions with a definitive pathological diagnosis, the sensitivity for the diagnosis of GIST was 82% and the specificity 100%[41].

ROLE OF THE NUCLEAR MEDICINE SPECIALIST

PET with fluorodeoxyglucose (FDG-PET) is highly sensitive detection of very metabolically active tumors resulting from a significant glycolysis (See Figure 6); however, this test is not considered to be specific enough to obtain a preoperative diagnostic, so it has not replaced CT as the initial imaging modality of choice in patients suspected of having a mesenchymal tumor in the GI tract. FDG-PET may be useful for detecting an unknown primary site or resolving ambiguities on the CT (inconclusive CT findings or inconsistent with clinical findings)[42]. The reported sensitivity of PET for GIST (including metastatic lesions) is 86%-100%[43].

The FDG-PET response, which is characterized by a mark in the glycolytic metabolism of tumors, may be seen one month after starting treatment with Imatinib-Mesylate and an early response may be seen in the first 24 h[44]. FDG-PET may detect an early response to a tyrosine kinase inhibitor, which may be important when the treatment is administered and would allow identification of patients with primary resistance to treatment or even identify secondary resistance in the case of patients already treated with Imatinib-Mesylate[45].

ROLE OF SURGEON

Surgery is the only potentially curative treatment for

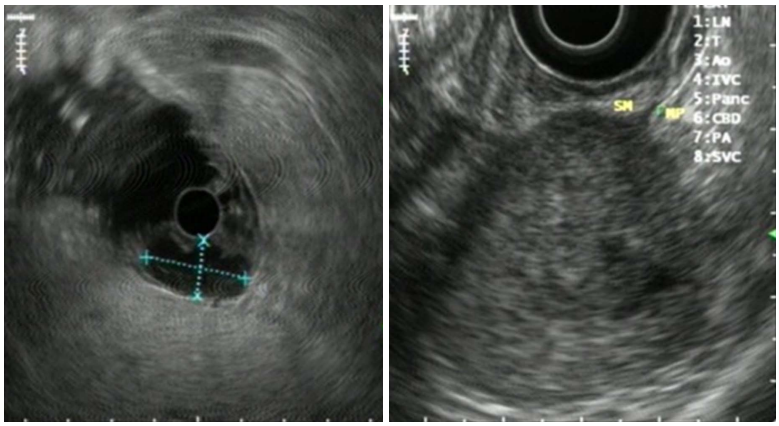

Figure 5 Endoscopic ultrasonography images of gastrointestinal stromal tumors.

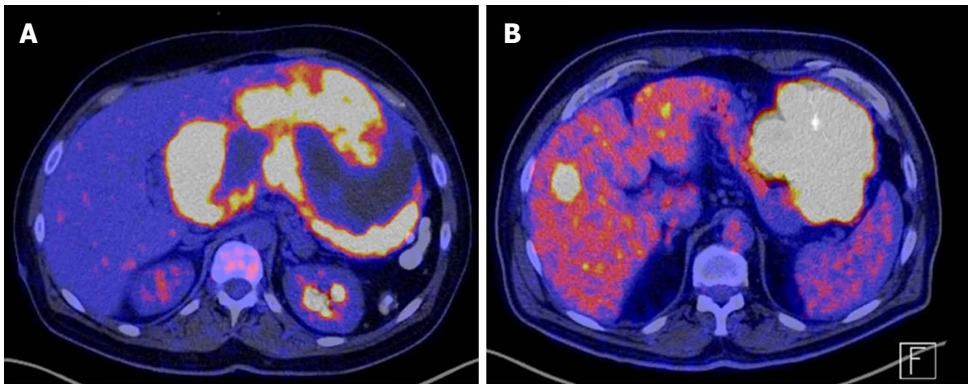

Figure 6 Gastrointestinal stromal tumors on positron emission tomography with fluorodeoxyglucose. A: Giant gastric GIST on a patient with neurofibromatosis type 1; B: Gastric GIST with an unique liver metastasis. GIST: Gastrointestinal stromal tumor.

suspected resectable GIST. The primary objective of this process is to ensure that clear resection margins are obtained in a complete resection of the tumor, and it can be extirpated without tumor pseudocapsule rupture; nevertheless wide margins have no benefit in disease control[5]. Conservative surgery must be the procedure of choice due to local GIST infiltrative behavior; lymphadenectomy is not necessary due to lymphatic affectation, which is rare[46]. A thorough exploration of the liver and parietal peritoneum is important in order to objectify possible metastases.

At least 40%-50% of patients who have undergone optimal surgery may experience a tumor recurrence; however, with the appearance of TKI, a new option disease control has been offered[47,48] (See Figure 1).

The management of GISTs < 2 cm is controversial; in spite of the fact that an active follow-up of the lesion could be an option, surgery should be considered because there is no data concerning growth behavior and metastasic potential[49,50].

RESACTABLE DISEASE

Stomach

In the case of gastric GISTs, which is the most common location, wedge resections are preferred to classic gastrectomies[51]. Currently, there are multiple studies comparing laparoscopic wedge resection for gastric GISTs versus open surgery showing multiple benefits that patients could obtain resulting from this less invasive approach as reducing postoperative discomfort or shortening the length of postoperative hospital stay. In this sense, laparoscopy wedge resection is considerate the standard treatment for gastric GIST[52-55]. In a multi-institutional analysis performed by Bischof *et al*[56], minimally invasive surgery for gastric GIST was shown to reduce length of the hospital stay, blood loss, and morbidity with same R0 and tumor rupture rates. The main limitation to laparoscopy resection is technical difficulty due to tumor size and location; nevertheless, multiple studies have demonstrated that laparoscopy wedge resection for gastric GISTs > 5 cm is feasible[54,57,58].

Incidental GIST finding in bariatric surgery is rare with a reported incidence between 0.3%-1.2% in different series of sleeve gastrectomy and gastric bypass[59-61]. In many cases, the performed procedure may be curative; however, the finding of GISTs in certain locations such as the gastroesophogeal junction or lesser curve could require abandoning the proposed technique in favor of a suitable and complete tumor excision[59].

Small bowel

For patients who have primary localized small bowel GIST, segmental bowel resection remains the first choice of treatment. Emergent resections are more often needed in patients diagnosed with small bowel stromal tumors secondary to hemorrhage, obstruction, or perforation[62]. Laparoscopic segmental resection with intra- or extracorporeal anastamoses, when possible, is the elective approach achieving comparable oncologic results[63-65]. Tabrizian *et al*[66] reported that in their series, laparoscopic removal of tumors up to 85 mm shows low rates of morbidity (10%), mortality (1.3%), and conversion (19%); the main reason for conversion was the tumor's proximity to the gastroesophageal junction, local invasion of adjacent organs, association with another malignant lesion, preoperative tumor perforation, extensive adhesions, and large tumor size[66].

Colon

In spite of the fact that the colon is an uncommon location for GISTs, patients with tumors in this location present a much poorer prognosis with a higher rate of disease-specific mortality and a higher percentage of patients with distant disease[67,68].

Due to intrinsic characteristics of GIST tumors, a wide resection is not required and segmental colectomy is the standard approach; Moreover, as previously mentioned, GIST does not metastasize through lymphatics thus mesocolic resection is unnecessary[69].

GISTs in complex anatomical locations

In cases of potentially resectable disease that are located in complex anatomic locations and require extensive organ disruption, neoadjuvant therapy with Imatinib has been proposed to downstage tumors and facilitates complete resection or decrease the morbidity of resection[67].

Esophagus: Esophageal location of a GIST is infrequent[16]. Robb *et al*[14] proposed that enucleation of the tumor is safe for esophageal GISTs < 65 mm as long as negative margins and intact pseudocapsule can be achieved, while in tumors of > 90 mm with evidence of mucosal ulceration and/or a high mitotic activity, an esophagectomy should be performed. The choice between esophagectomy and enucleation for tumors of between 65 and 90 mm needs further clarification with the decision being influenced by the location, malignant risk, patient comorbidity, and the presence of mucosal affectation[14,70]. Neoadjuvant therapy is indicated with the aim of shrinking the primary tumor[70].

Duodenum: GISTs located in duodenum are rarely observed and often manifest as nonspecific abdominal pain, gastrointestinal hemorrhaging, and intestinal obstruction may infrequently have been observed[71,72].

When possible, limited resection should be the procedure of choice; nonetheless, due to peculiar anatomic location because of proximity of the pancreatic head and papilla of Vater and the difficulties to get an adequate section margin, Pancreaticoduodenectomy is often necessary[73]. In the meta-analysis accomplished by Chok *et al*[74], it was appreciated an increase of positive margins in case of Limited resection, although there was no significant difference in local recurrence between Limited Resection and Pancreaticoduodenectomy.

Neoadjuvant treatment with TKI has been proposed to downstage GISTs, and possibly increases the chance of preserving normal biliary and pancreatic anatomy which would otherwise require more aggressive surgery[74,75].

Rectum: Outcomes in colonic or rectal locations appear to be worse than those located in the stomach[67]. Rectal GIST may require an abdominoperineal amputation to achieve a surgically complete resection. To avoid an extensive and limiting surgery, neoadjuvant Imatinib-Mesylate should be considered to reduce tumor size and facilitate complete surgical resection by increasing negative margins and less radical sphincter-sparing surgery[76,77]. Laparoscopic sphincter-preserving surgery is safe and feasible after neoadjuvant treatment of Imatynib Mesylate[78]. Mesorectal resection for rectal GISTs is not required due to the absence of lymphatic dissemination. Transanal endoscopic surgery has been employed for local treatment of low rectal GISTs with no evidence of recurrence after an 18-mo follow-up[79].

LOCALLY ADVANCED DISEASE OR BORDERLINE RESECTABLE

Most studies define locally advanced primary GIST as the significant involvement of a single organ with large tumor size or extension of the tumor to adjacent organs[80]. Neoadjuvant use of imatinib has been demonstrated to be useful in primary locally advanced GISTs by causing a decrease in tumor volume in the majority of the patients. Tumor response may facilitate complete resection of these advanced tumors and could allow less invasive procedures without tumor rupture[10,16,81].

METASTATIC AND RECURRENT DISEASE

Metastases may be detected at first presentation or at the time of disease progression. The first line of treatment for patients with metastatic or recurrent GISTs are TKI in the form of neoadjuvant or adjuvant therapy with the choice of treatment being Imatinib-mesylate[82]. The appropriate time for surgical intervention is still unknown. It is proposed to consider surgery if a complete cytoreductive resection is feasible after six to nine months with a tyrosine kinase inhibitor.

After treatment with TKI, there are three possible radiological response of the disease[83]: (1) Complete response: Non metabolic activity in PET; Disappearance

of disease in CT; Infrequent clinical situation. (2) Partial response or responding to drug therapy; Decrease of metabolic activity in PET and/or decrease of size in CT. (3) Stable disease: Disease is radiographically (CT, PET) stable. (4) Limited/localized disease progression: Progression in spite of drug therapy is seen at one or a few (but not all) sites of disease. Patients with Partial response, stable disease or limited/localized disease progression could undergo cytoreductive surgery. And (5) Generalized disease progression: In this case, disease is progressing at multiple sites while on drug therapy. Debulking surgery does not seem to prolong survival so cytoreductive surgery is not recommended.

Peritoneum metastases; "GISTosis"

Peritoneal GIST metastases may be detected, especially in cases in which the primary tumor ruptured spontaneously or surgically. When disease progression occurs due to Imatinib resistance and GISTs relapse loco-regionally after surgical resection or disease disseminates to peritoneum, prognosis is poor and standard treatments such as conventional surgery, radiotherapy ,and systemic chemotherapy are generally ineffective[84].

Imatinib-Mesylate and Sunitib-Malate (used in cases in which GIST develops resistance to Imatinib) have been shown to increase disease control and survival rates; nevertheless, although patients experience durable periods of disease stability to Imatinib lasting months to years, the response is not maintained indefinitely[85].

Aggressive surgical procedures to treat loco-regional relapse and peritoneal metastases have been proposed. These cytoreductive strategies involve peritonectomy procedures and multivisceral resection to remove all macroscopic tumor(s)[84]. Cytoreductive surgery has been shown to increase progression-free survival and overall survival rates in patients with metastatic GIST who are receiving Imatinib Mesylate therapy. Patients with stable disease or responsiveness to Imatinib Mesylate had demonstrated an increase in survival rates compared to those with disease progression[81,85-87].

Sugarbaker[84] proposes the complete resection of recurrent sarcomas using peritonectomy and visceral resections to complete cytoreduction of disease and perio-perative intraperitoneal chemotherapy. The randomized trial performed by Bonvalot *et al*[88] in 2005 demonstrate the importance of the positive impact of complete cytoreductive surgery; however, the use of intraperitoneal chemotherapy didn't increase greatly overall survival of sarcomatosis. Cytoreductive Surgery combined with perioperative intraperitoneal chemotherapy is a promising future approach to sarcomatosis, awaiting new chemotherapy agents.

After the surgical excision of GISTs metastases, it is necessary to continue treatment with TKI[85].

Liver metastases

GIST metastases are often located in liver and may appear as primary disease or as a recurrence after surgery. In cases of metastatic GIST in which stable disease or localized disease progression exists, hepatic resection is the mainstay of treatment for liver metastasis[83].

In DeMatteo *et al*[89] 56 patients with liver metastasis who underwent complete resection of all gross disease had significantly longer survival (1-,3-,5-year disease-specific survival rate was 88%, 50%, and 30%, respectively) than those 275 patients who did not undergo complete resection (1-, 3-, and 5-year disease-specific survival rates of 50%, 13%, and 4%, respectively). Completing surgical treatment with Imatinib Mesylate showed an increase in disease-free and overall survival[37,90].

There exist few references in the literature concerning liver transplantation in patients with metastatic sarcoma showing uninspiring results[91,92].

ROLE OF ONCOLOGIST

The use of TKI against GIST introduced a new era in molecular-targeted therapies in clinical oncology. The oncologist plays a major role in GIST treatment for carrying out the indication for the TKI for metastatic tumors after a curative surgery or as a neoadjuvant treatment (See Figure 1).

Almost 85% of GISTs have a mutation in KIT or PDGFRA that induces an KIT activation, which is a tyrosine kinase receptor that stimulates the growth of cancer cells. Mutational analysis is acquiring a growing importance and should be performed when adjuvant and/or neoadjuvant therapy show possible mutations with a tendency toward Imatinib Mesylate-resistance[17]. Tyrosine kinase inhibitor selection based on gene mutations is described in Table 1.

KIT gene mutations (80%): KIT exon 11 is the most common mutation and may be observed in approximately 75% of all mutation-positive tumors primarily affecting codons 557-559. These mutations are most commonly observed in gastric GIST. In the Z9001 trial, patients with exon 11 mutation proved to experience greater benefits from adjuvant Imatinib with higher rates of relapse-free survival although these mutations indicate poorer prognosis and high metastatic risk[17,93,94]. Exon 9 mutations (approximately 10%) are associated with poor Imatinib response. Mutations in exons 8, 13, and 17 are infrequent and seem to be < 3%[24].

PDGFRA gene mutations (5%-8%): GISTs with PDGFRA mutations are regularly located in stomach[17]. The D842V mutation in PDGFRA exon 18 is the most common mutation found (65%-75% of PDGFRA mutations)[94]; this mutation is associated with Imatinib and Sunitinib resistance[95,96]. Non-D842V exon 18, 12, and 14 mutations are rare and sensitive to Imatinib.

Table 1 Tyrosine kinase inhibitor election based on genes mutations

Gene	Mutation	TKI. Dose
KIT	Exon 11	Imatinib-Mesylate 400 mg/d
	Exon 13	
	Exon 17	
	Exon 9	Imatinib-Mesylate 800 mg/d
PDGFRA	Exon 18. D842V mutation	Sunitinib 50 mg/d
		Regorafenib 160 mg/d
	Exon 12	Imatinib-Mesylate 400 mg/d
	Exon 14	
	Exon 18. Non D842V mutations.	
Wild-type		Sunitinib 50 mg/d
		Regorafenib 160 mg/d

Wild-type GISTs (12%-15%; 90% of pediatric GISTs): In these cases, there are no detectable mutations in KIT or PDGFRA genes that are resistant to treatment with Imatinib although tyrosine kinases are still activated. Wild-type GISTs represent a heterogeneous group that includes several oncogenic mutations such as BRAF V600E substitution, NF1 mutation, and defects in the succinate dehydrogenase complex[97-99]. Second line TKI are recommended despite poor response by these tumors.

KIT-negative GISTs (CD117-negative): Approximately 5% of GISTs do not express CD117 by immunoreactivity but 30%-50% of cases have KIT or PDGFRA mutations[100].

When patients present primary or secondary resistance to Imatinib, second-line treatment with Sunitinib and third-line treatment with Regorafenib are recommended.

ADJUVANT THERAPY

In spite of performing a complete resection of the tumor without tumor rupture and appearance of negative margins, GISTs still have some malignant potential and may recur or metastasize. It is necessary to identify those patients who may derive benefits from the adjuvant Imatinib-Mesylate because of their high risk of recurrence or metastases following resection. Several risk stratification models have been proposed to estimate the risk of recurrence and identify high risk GISTs after resection, so the indication of adjuvant Imatinib can be individualized. In multiples models, the main predictors of recurrence established were tumor mitotic rate, size, and location[47,101]. Increased tumor size, high mitotic activity, or extragastric location such as small bowel, colon, rectum, or mesentery is associated with an increased risk of poor outcomes. The oldest risk stratification model is the consensus from the National Institutes of Health (NIH) which stratifies risk on the basis of tumor size and mitotic count and has demonstrated its usefulness in predicting GIST behavior[8,102]. On the other hand, Miettinen *et al*[103] emphasized the importance of location to the risk of recurrence. The revised NIH consensus criteria by Joensuu *et al*[104] in 2008 included the presence of either spontaneous tumor rupture or that occurring, which worsens the prognosis and location because of the better prognosis of gastric location versus extra gastric GISTs (Table 2)[105]. Incomplete resection has demonstrated to adversely affect overall survival (OS)[106].

In a phase II US Intergroup trial ACOSOG Z9000[107], 106 patients with resected high-risk GIST were included. High risk was defined as tumors >10 cm, evidence of capsular rupture, hemorrhage, or multifocal disease with > 5 tumor foci. Patients were treated after a complete resection with daily oral 400 mg Imatinib for one year. The primary endpoint was OS with 1-, 2-, and 3-year OS of 99%, 97%, and 83%, respectively. One-, 2-, and 3-year recurrence-free survival was 96%, 60%, and 40%, respectively.

In the ACOSOG Z9001 randomized phase III multicenter trial, 713 patients with complete gross resection of a primary GIST at least 3 cm in size and showing positive staining for KIT protein were randomly assigned to one year of adjuvant Imatinib (400 mg daily) or placebo[108]. Primary endpoint was recurrence-free survival (RFS). Imatinib was shown to increase RFS compared with placebo (98% versus 83% at one year; hazard ratio [HR] 0.35; $P < 0.0001$). Although no differences in the case of OS (99.2% *vs* 99.7% at one year; HR 0.66; $P = 0.47$), it was considered justified because of short follow-up time and the crossover study design, which allowed patients with tumor recurrence assigned to the placebo arm to receive Imatinib-Mesylate. In this study, patients with exon 11 mutations showed the longest progression-free survival (PFS), while those with an exon 9 mutation had the worst outcomes; however, those patients with exon 9 mutations treated with higher dose of Imatinib showed greater PFS.

In the EORTC 62024 phase III trial[109], 908 patients with intermediate- or high-risk GIST were included and assigned to two years of daily Imatinib 400 mg after complete resection compared to only surgery. The primary endpoint at the origin was OS; however, in 2009, the primary endpoint was changed to Imatinib failure-free survival (IFFS). With a median follow-up

Table 2 Risk stratification criteria for primary resectable gastrointestinal stromal tumor porposed by Joensuu

Risk category	Tumour size (cm)	Mitotic index (per 50 HPF)	Primary tumour site
Very low risk	≤ 2.0	≤ 5	Any
Low risk	2.1-5.0	≤ 5	Any
Intermediate risk	≤ 5.0	6-10	Gastric
	5.1-10.0	≤ 5	Gastric
High risk	Any	Any	Tumour rupture
	> 10.0	Any	Any
	Any	> 10	Any
	> 5.0	> 5	Any
	≤ 5.0	> 5	Non-gastric
	5.1-10.0	≤ 5	Non-gastric

HPF: High-power field.

of 4.7 years, 5-year IFFS was 87% in those patients treated with Imatinib versus 84% in the control arm (HR = 0.79; 98.5%CI: 0.50-1.25; P = 0.21); RFS was 84% in the Imatinib group *vs* 66% at 3 years and 69% versus 63% at 5 years (log-rank P < 0.001).

In the Scandinavian Sarcoma Group (SSG) XVIII trial comparing 12 mo *vs* 36 mo of adjuvant 400 mg/d Imatinib, 400 patients with high-risk complete resected GIST were included. Patients who were treated with 36 months of Imatinib showed an increase in RFS compared with those treated 12 mo (HR = 0.46; 95%CI: 0.32-0.65; P < 0.001). Five-year RFS was 65.6% in the 36-mo group compared to 47.9% in the 12-mo group. Patients treated with 36 mo of Imatinib showed an increase in OS (HR = 0.45; 95%CI: 0.22-0.89; P = 0.02) with 5-year survival of 92.0% versus 81.7% in those patients treated for 12 mo[110]. In the 36-mo group, it was observed that a high number of patients discontinued Imatinib for reasons other than GIST recurrence (25.8% *vs* 12.6%). In this study, patients with KIT exon 11 deletion mutations benefitted most from the 36 mo of adjuvant Imatinib, while in the other mutational subgroups examined there were no significant benefits[111].

Adjuvant treatment is recommended in those patients who have R0 primary high risk GISTs; however, the optimal indication of adjuvant treatment in high risk patients is not clear, so each case must be approached individually in multidisciplinary specialized committees that balance beneficial and negative impacts. The standard treatment for high risk GIST is adjuvant therapy of 400 mg/d of Imatinib-Mesylate over three years. Two randomized trials comparing prolonged adjuvant therapy with Imatinib-Mesylate versus standard treatment (five versus three years in SSG XXII [NCT02413736] and six versus three years ImadGist [NCT02260505]) exist.

NEOADJUVANT THERAPY

Neoadjuvant Imatinib-Mesylate should be, considered for shrinking the tumor in cases of locally advanced primary or recurrent disease, unresectable or potentially resectable metastasic tumors, and potentially resectable disease in complex anatomic locations to decrease the related morbidity[112]. There is no consensus about duration of the treatment with Imatinib-Mesylate; however, 3-12 mo of treatment with numerous imaging control studies would be an acceptable management[113]. Usually, maximal tumor response occurs after 4 to 12 mo of treatment[114].

Tumors located in complex anatomic locations such as the esophagus, duodenum, or rectum may show a major benefit of initial neoadjuvant treatment to produce less extensive organ disruption interventions[14,78,115].

Preoperative Imatinib has demonstrated to facilitate complete resection of locally advanced primary, recurrent, or metastatic GISTs. In Andtbacka *et al*[80], a series of 46 patients, who underwent surgery after neoadjuvant Imatinib, was retrospectively reviewed; 35 patients were treated for recurrent or metastatic GIST obtaining a complete resection in 11 patients. This study showed that those patients with a partial radiographic tumor response to neoadjuvant showed significantly higher complete resection rates than patients with progressive disease (91% *vs* 4%; P < 0.001).

In a study by Bonvalot *et al*[81], 22 of 180 patients with unresectable GIST treated with neoadjuvant Imatinib (19 received imatinib 400 mg/d and three received 800 mg/d) and no radiographic evidence of overall progression underwent surgery. There were five patients with metastases who underwent emergency surgery due to hemorrhaging and three of them died in the early postoperative period. When surgery was planned, 15 of 17 patients (88%) had a complete resection.

In the study by Chandrajit *et al*[30], those patients with advanced GISTs under neoadjuvant therapy with Imatinib showing stable disease or limited progression had an increase in OS rates after cytoreductive surgery.

The analysis realized by European Organization for Research and Treatment of Cancer-Soft Tissue and Bone Sarcoma Group (EORTC STBSG), which included databases from 10 EORTC STBSG sarcoma centers, indicated that the largest group of GIST patients (n = 161) were treated with neoadjuvant Imatinib. The most

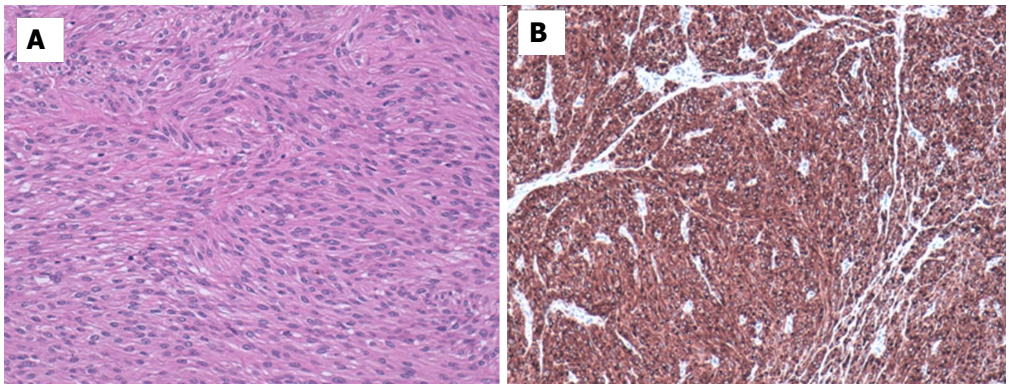

Figure 7 Histological sections of ileal gastrointestinal stromal tumor. A: HE stain; B: Immunohistochemistry with C-KIT.

Table 3 Items that pathology report should include

Pathology report items
Localization
Size
Number of foci
Tumor infiltration
Histologic subtype
Depth of tumor infiltration
Grade of dedifferentation
Mitotic rate
Ki67
Grade of necrosis
Grade of hemorrhage
Margins
Staging
Mutational study

common location was the stomach (55%) followed by rectum (20%), duodenum (10%), ileum/jejunum/other (11%), and esophagus (3%). Median time on therapy with neoadjuvant Imatinib was 40 weeks, and R0 was obtained in 83% of patients. During follow-up, they observed 37 disease recurrences (23%) and only five patients (3%) presented a local relapse with a 5-year DFS rate of 65% (95%CI: 59.1%-70.9%). Five-year OS was 87% (95%CI: 78%-98%), and median OS was 104 mo. Patients who continued with Imatinib after surgery presented better rates of DFS.

In cases of patients with metastasic or recurrent disease under treatment with second-line sunitinib or third-line regorafenib, the role of debulking surgery is still not clear, and there is only a certain amount of information concerning emergency interventions. It is necessary to bear in mind that in these cases the tumors are advanced and resistant to standard treatment, so the potential benefit of the surgery is not known[112].

ROLE OF PATHOLOGIST

Pathologic diagnosis has a major impact on GIST management, both at the preoperative time and after complete surgical resection. Data obtained by pathologists need to be stratified according to risk, and prognoses and possible therapies based on primary and acquired secondary resistance to TKI need to be determined.

GISTs in GI tracts are normally found in the subepithelial layer; however, as they become larger, they may cause epithelial ulceration. Currently, GIST's pathological diagnosis depends on the combination of morphology, immunohistochemistry (CD117 and/or DOG1), and molecular analysis (See Role of Oncologist).

Morphologically, GISTs are subdivided into spindle (70%), epithelioid (20%), and mixed-type cells, but it is considered that cell type influence on the outcome is not relevant[8]. GIST may be divided into eight different subtypes[116]: (1) Spindle cell subtypes: sclerosing, palisading-vacuolated, hypercellular and sarcomatous spindle cell; and (2) Epithelioid cell subtypes: sclerosing, discohesive, hypercellular and epithelioid spindle cell.

The distinction between benign and malignant depends on the presence of nuclear atypia and presence of necrosis, hemorrhaging, and mitotic activity. It is necessary to determine mitotic rate, grade of dedifferentiation, size, location, tumor infiltration, grade of necrosis and hemorrhage, surgical margins, and whether a tumor ruptures because these factors are implicated in the risk of relapse[117]. Ki67 is an important prognostic factor that has been implicated in recurrence and survival and should be included in pathologist's report[118,119] (See Table 3). The depth of tumor infiltration, including serosal penetration has been proposed as a prognostic factor for patients with GISTs with significantly poorer prognosis compared to its absence[120,121].

Most GIST (> 90%) shows overexpression of the receptor tyrosine kinase KIT (CD117) by immunohistochemistry. On the other hand, a proportion of GISTs (near 5%) which are CD117-negative exists; however, approximately one third of these cases stained with discovered on GIST (DOG)1, which is expressed strongly on GIST and is rarely expressed on other soft tissue tumors[122,123] (See Figure 7). PKC-θ has lower specificity than DOG-1, but it may be a useful biomarker when combined with DOG1. Using both as an important diagnostic tool in the diagnosis of KIT-negative GISTs, even in wild type GISTs may prove useful for diagnosing

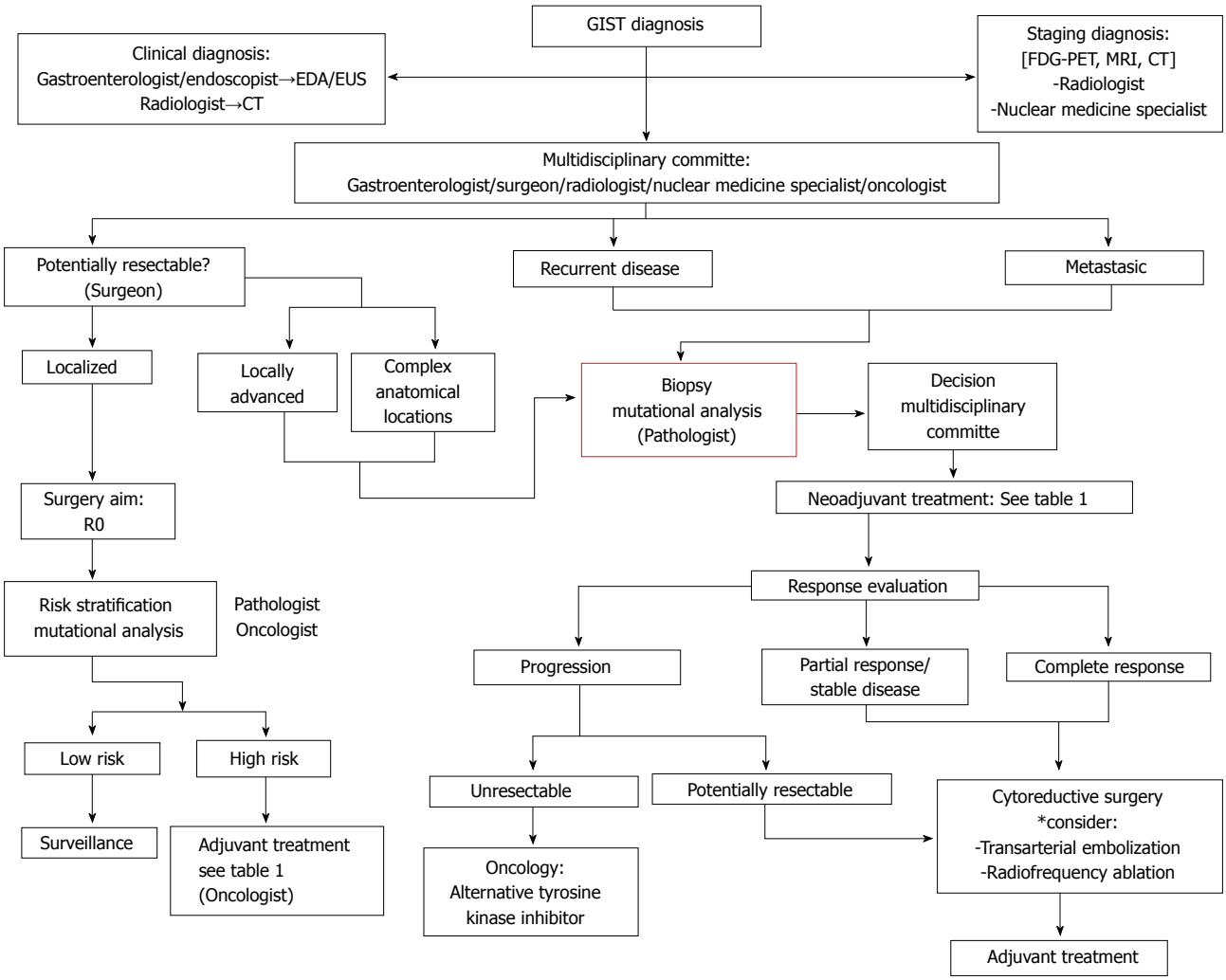

Figure 8 Management algorithm of gastrointestinal stromal tumors. GIST: Gastrointestinal stromal tumor.

GIST[124].

GIST's mutational study is becoming increasingly important. Mutational analyses allow correlations of sensitivity or resistance to molecular-targeted therapies and doses. These types of analyses have prognostic value, so that they play a major role in GIST management[122] (see Role of Oncologist).

CONCLUSION

The diagnosis of GIST has increased in recent years thanks to new imaging techniques which have increased the interest in the management of this type of tumors.

The clinical diagnosis is based on the CT, EDA and/or endoscopic US and staging diagnosis is obtained by CT and FDG-PET. The histological diagnosis is based on US-guided biopsy or percutaneous biopsy prior to surgery; In case of high suspicion in the imaging tests, surgical resection without previous biopsy would be justified.

The biological behavior of the GIST is explained according to the mitotic index, Ki67, anatomical location, size and mutational status.

Surgical resection with free margins of tumor disease R0 is the only potentially curative therapeutic option.

Therapies with TKI (Imatinib, Sunitinib and Regorafenib) have let a noteworthy improvement in the rates of disease-free survival and overall survival, even in recurrent or unresectable metastatic GISTs.

GISTs are the paradigm of a cancer with molecular targeted therapy and its management requires a multi-disciplinary approach (See algorithm of management in Figure 8).

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REVIEW

New therapeutic options opened by the molecular classification of gastric cancer

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Abstract

Gastric cancer (GC) is one of the most lethal and aggressive cancers, being the third cause of cancer related death worldwide. Even with radical gastrectomy and the latest generation of molecular chemotherapeutics, the numbers of recurrence and mortality remains high. This is due to its biological heterogeneity based on the interaction between multiple factors, from genomic to environmental factors, diet or infections with various pathogens. Therefore, understanding the molecular characteristics at a genomic level is critical to develop new treatment strategies. Recent advances in GC molecular classification provide the unique opportunity to improve GC therapy by exploiting the biomarkers and developing novel targeted therapy specific to each subtype. This article highlights the molecular characteristics of each subtype of gastric cancer that could be considered in shaping a therapeutic decision, and also presents the completed and ongoing clinical trials addressed to those targets. The implementation of the novel molecular classification system will allow a preliminary patient selection for clinical trials, a mandatory issue if it is desired to test the efficacy of a certain inhibitor to the given target. This will represent a substantial advance as well as a powerful tool for targeted therapy. Nevertheless, translating the scientific results into new personalized treatment opportunities is needed in order to improve clinical care, the survival and quality of life of patients with GC.

Key words: Gastric cancer; Molecular classification; Immunotherapy; Targeted therapy; Clinical trials

Core tip: A new molecular classification of gastric cancer (GC) became available after publication of three landmark studies: The Cancer Genome Atlas, "Singapore-Duke" study, and Asian Cancer Research Group, allowing patient selection for specific targeted therapy. The Epstein-Barr virus positive, microsatellite stable TP53-active or microsatellite-unstable tumors subtypes presents tumour infiltrating patterns with overexpression of PD-1, PD-L1, PDL-2. Preliminary data show high response rate to immunotherapy and open new avenues to gene therapy. Currently effective therapies for diffuse GC are lacking. Mutations in e-cadherin and Ras homolog family member A genes, or Claudin-18-ARHGAP6/26 fusions may be exploited as therapeutic targets. The only targeted therapies approved so far for chromosomal instability and microsatellite stable TP53-inactive subtypes of GC are trastuzumab and ramucirumab (HER2 and VEGFR2 inhibitors).

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INTRODUCTION

Gastric cancer (GC) is a common and deadly cancer worldwide, with over 1000000 patients being diagnosed and over 723000 dying each year[1]. Five-year survival for advanced or metastatic GC is 5%-20%, and median overall survival less than 1 year[2]. As in all cancer subtypes, angiogenesis, fibrosis and inflammation are critical processes in local progression and metastasis. These factors do so in part by creating a tumour microenvironment that is characterized by hypoxia and immunosuppression, which thwarts immune system' s ability to fight the cancer. As a result, no single chemotherapy or molecularly targeted drug, or even combination regimen, consistently leads to objective and durable tumour shrinkage in GC.

Surgical resection still represents the only potentially curative treatment in gastric cancer patients. However, in most cases, patients are diagnosed with advanced disease and therapeutic approach, include, beside surgery, adjuvant or neoadjuvant chemotherapy and radiotherapy. Molecular targeted therapy in advanced gastric cancer it is currently limited to trastuzumab, as first-line therapy in patients with HER-2 positive tumours and ramucirumab, alone or in combination with chemotherapy, as second-line therapy[3].

In the last years, several studies have attempted, on the basis of microarray and meta-analyses, to highlight a genetic signature for gastric cancer linked to either tumour stage[4,5] or prognosis[6,7]. Furthermore, those genetic signatures were the basis for further studies of targeting and inhibition by means of RNA interference technology of overexpressed genes in order to validate them as therapeutic targets in gastric cancer[8-10].

An important step in obtaining more effective therapeutic responses is identifying subsets of patients that are suitable candidates to benefit from specific therapeutic agents, knowing that differences in gene expression profile are correlated with different treatment response[11,12]. This has become possible in gastric cancer due to results from three landmark studies that provided new genetic and epigenetic molecular classifications of GC: the Cancer Genome Atlas (TCGA), "Singapore-Duke" study, and Asian Cancer Research Group (ACRG)[1,13,14]. These findings are offering an unprecedented opportunity to make substantial progress in GC therapy. Moreover, based on the promising results obtained in other solid tumors[15], new treatment strategies, like immunotherapy, are emerging in the field of gastric cancer therapeutics. Preliminary results showed that targeting the immune checkpoint pathways may represent a promising strategy that can lead to better outcomes in gastric cancer patients.

NEW MOLECULAR CLASSIFICATIONS OF GASTRIC CANCER

Traditionally, gastric cancers were histologically divided into main types: intestinal and diffuse, according to Lauren classification[16]. In 2010, World Health Organization (WHO), distribute gastric carcinomas in four groups of tumors: papillary, tubular, mucinous and poorly cohesive. Beside adenocarcinoma, the WHO classification also included less frequent histologic variants[7].

Latest advances in molecular methods such as next-generation sequencing (NGS), including DNA sequencing, RNA sequencing, whole-exome sequencing, copy number variation analysis, and DNA methylation arrays, have increased our understanding of GC biology, and led to the development of a new comprehensive molecular classification.

One of the first studies aimed to identify molecular gastric cancer subtypes was the study undertaken by Tan *et al*[17], who analysed patterns of gene expression for 28 gastric cell lines and proposed two major distinct subtypes of GC: intestinal (G-INT) and diffuse (G-DIF) that overlaps with Lauren's intestinal or diffuse-type. These intrinsic subgroups were validated in 4 independent cohort totalized 521 primary tumors. Moreover, the authors showed that the G-INT cell lines are more responsive to treatment with 5-fluorouracil and oxaliplatin but more resistant to cisplatin than the G-DIF cell lines, and the patients with G-INT cancers have better overall survival in comparison to patients with G-DIF tumors.

In 2013, in a subsequent study Lei *et al*[18] using consensus hierarchical clustering with selection by

repetitive features on 248 gastric tumors identified three subtypes of gastric adenocarcinoma: mesenchymal, proliferative and metabolic. Each subtype display distinct genomic and epigenetic properties and drug sensitivities. The proliferative subtype includes tumors with a high level of genomic instability, DNA hypomethylation and TP53 mutations. For this tumour subgroup, frequent mutations in CCNE1, MYC, ERBB2 and KRAS genes, were also described. The metabolic subtype comprises cancer cells with a gene expression pattern similar to cells from normal mucosa that are more sensitive to 5-fluorouracil treatment than mesenchymal or proliferative tumors subtypes. Tumors in the mesenchymal subtype contain cells with stem cell-like properties, with high activity of the epithelial-mesenchymal transition pathway. The *in vitro* studies show that the cell lines of this subtype are sensitive to PI3K/Akt/mTOR inhibitors.

In 2014, as part of The Cancer Genome Atlas (TCGA) project, Adam Bass *et al*[1] realize a comprehensive molecular characterization of 295 primary gastric adenocarcinomas and proposed a new molecular classification system for gastric cancer which comprises four subtypes: tumors positive for Epstein-Barr virus (EBV), microsatellite unstable tumours (MSI), genomically stable tumours (GS) and tumours with chromosomal instability (CIN).

A similar approach had researchers from Asian Cancer Research Group (ACRG), who analysed gene expression data from 300 primary gastric tumors. Their findings have led to a novel proposal of gastric cancer molecular classification that includes four tumors subtypes: with microsatellite stability (MSS)/epithelial-mesenchymal transition (EMT), microsatellite-unstable tumors (MSI), microsatellite stable TP53-active (MSS/TP53+) and microsatellite stable TP53-inactive (MSS/TP53-)[14].

Both molecular classification systems highlight the main molecular alterations specific to each subtype, together with their frequency which can provide a new orientation in targeted therapy. In addition, the ACRG classification model provides useful information about disease progression and prognosis.

Although there are not equivalent, the subgroups proposed by the two research teams share common features and are partially overlapping. The similarities were observed between MSI subtypes, the MSS/TP53+ and EBV positive subgroups, the MSS/EMT subtype and the GS subgroup, and also in the MSS/TP53- and CIN. Figure 1 presents the major features and genomic alterations associated with each GC subtype according to TCGA and ACRG studies.

The EBV-infected tumours represents around 9% of GC according to TCGA classification and are characterized by high level of DNA hypermethylation, non-silent mutations in phosphatidylinositol 3-kinase PIK3CA (80% of the current subtype cases), AT-rich interactive domain-containing protein 1A (ARID1A) (54%), B-cell lymphoma 6 Corepressor (BCOR) (23%), and recurrent amplification at 9p24.1, a chromosomal region that contains Janus-associated kinase 2 (JAK2) gene and two other genes that encodes for programmed death-ligand 1 and 2 (PD-L1, PDL-2) proteins (15%)[1,19].

The EBV subtype have some overlaps with the MSS/TP53+ subtype. The microsatellite stable TP53 active subtype appears to have a greater prevalence of APC, ARID1A, KRAS, PI3KCA and SMAD4 mutations compared with MSS/TP53- subtype and presents an intermediate rate of relapse and prognosis. All of these genetic alterations may have therapeutic value and must be exploited for the treatment of GC patients.

The MSI subtypes are mainly associated with hypermethylation of the MutL homolog 1 (MHL1) promoter, one of the genes involved in DNA mismatch repair (MMR) system. Due to MMR mechanism deficiency, this GC subtype has the highest rate of mutations compared to the others. Frequent recurrent mutations were observed in PIK3CA, ARIDA1, Erb-B2 receptor tyrosine kinase 3 and 2 (ERBB3, ERBB2), and epidermal growth factor receptor (EGFR) genes[1,20,21]. In the TCGA cohort, this subtype was associated with 23% of tumors and moreover with advanced age, female gender and less advanced tumoral stages. According to ACRG classification, the MSI group (22%) present recurrent mutations in KRAS, ALK, ARID1A, ERBB2, ERBB3 genes as well in genes involved in PI3K/PTEN/mTOR signaling. Usually occurs in the antral region and have the lowest recurrence rate (22%) and the best prognosis from all subgroups.

The GS subtype correspond to MSS/EMT subtype in that early age of appearance, association with diffuse type of GC and displaying low frequency of mutations compared to other gastric cancer subtypes. In the TCGA cohort 21.56% of cases were associated with GS subtype. Mutations in E-cadherin (CDH1) and Ras homolog family member A (RHOA) genes, together with the fusion between Claudin-18 (CLDN18) and Rho GTPase activating protein-6 or 26 (ARHGAP6, ARHGAP26), are the main feature of GS subtype of GC. The same genomic alterations were associated with MSS/EMT subtype in the ACRG classification, which represents 15.33% of cases. It has the highest recurrence rate (63%) and the worst prognosis among the four subtypes.

CDH1 mutations and EMT are representative features of this GC subtype. Both somatic and germline mutations, were identified. Somatic mutation have been detected in approximately 30% of GC and were related to poor prognosis[22]. Germline alterations in CDH1 gene are the main cause of hereditary diffuse gastric cancer and occur in about 40% of patients with this pathology[23]. E-cadherin, which is encoded by the CDH1 gene, is an adhesion molecule widely involved in carcinogenesis. E-cadherin deficiency has been linked to early tumor initiation in a large proportion of diffuse GC like signet ring adenocarcinoma, which is very resisting to all therapies, and hereditary diffuse GC, both with very poor survival[24,25].

Another hallmark of GS subtype are mutations in RHOA gene. RHOA is known to modulate Rho signalling

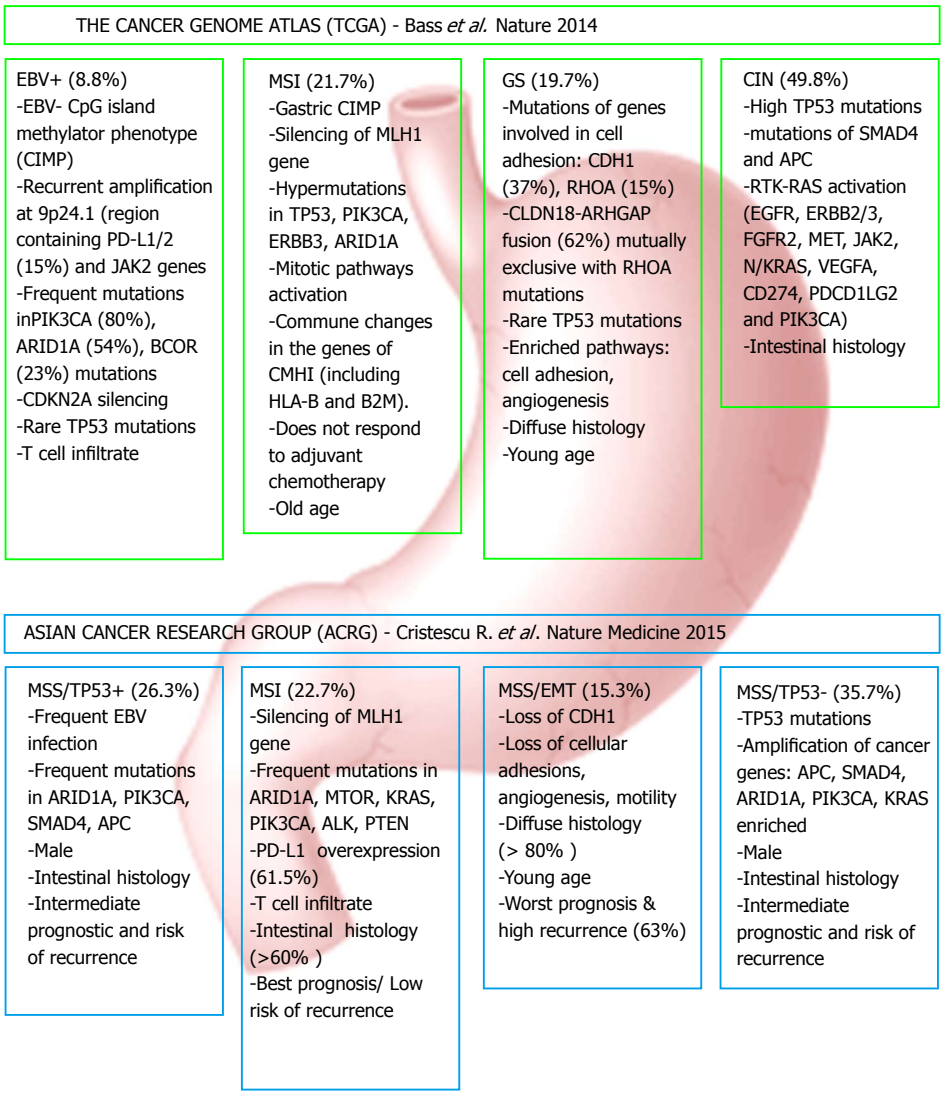

Figure 1 Molecular classification of gastric carcinoma: Molecular alteration and major features were associated with each subtype according to ATGC and Asian Cancer Research Group studies. EBV+: Epstein Barr virus positive; MSI: Microsatellite instable; GS: Genomic stable; CIN: Chromosomal instability; MSS: Microsatellite stable; TP53: Tumour protein 53; EMT: Epithelia-mesenchymal transition.

downstream effectors and its mutation can prevent programmed cell death[26]. In activated form, RHOA controls actin-myosin-dependent cell contractility and motility, and its mutations promote a diffuse growth pattern.

The third characteristic feature of this subtype is the fusion between CLDN18-ARHGAP6/ARHGAP26, a GTPase-activating protein that facilitates conversion of RHO GTPases to GDP. A recent study reported that in gastric epithelial cells, CLDN18-ARHGAP26 fusion might be involved in the epithelial-mesenchymal transition and cancer progression[27].

In genomically stable tumours, RHOA mutations and CLDN18-ARHGAP6 or ARHGAP26 fusions are mutually exclusive. Thereby, RHOA gene mutations and the gene fusions specific for GS subtype could become therapeutic targets in this group of gastric adenocarcinoma.

CIN tumors represent 45% of TCGA cases and are frequently located at the gastroesophageal junction/cardia region. Are commonly related to intestinal type of gastric cancer and are associated with MSS/TP53- subtype from ACRG classification. The CIN tumors display genomic amplifications of receptor tyrosine kinases (RTKs) from ERBB, vascular endothelial growth factor (VEGF), phosphatidylinositol-3-kinase (PI3K)/Akt, mammalian target of rapamycin (mTOR) and Met/hepatocyte growth factor (HGF) signaling pathways. Recurrent amplifications of cell cycle mediators: Cyclins E1, D1 (CCNE1, CCND1) and cell division protein kinase 6 (CDK6), VEGFA amplification, frequent TP53 mutation and high levels of phosphorylated epithelial growth factor receptor (EGFR) were also described for CIN tumors[1]. In the ACRG classification, the correspondent microsatellite stable TP53-inactive (MSS/TP53-) subtype - include tumours with high-grade aneuploidy, with frequent focal amplifications in Mouse double minute 2 homolog (MDM2), MYC, ERBB2, EGFR, CCNE1 and CCND1 genes and the highest TP53 mutations

Table 1 Selected active clinical trials involving molecular targeted therapies for specific gastric cancer subtypes

GC subtype	Molecular target	Therapeutic agents	Clinical trial name (ID)	Phase	Patients	Aditional treatments	ESCD	Clinical efficiency			Condition	Study (citation)
								ORR	mOS	mPFS		
EBV	PD-1	Pembrolizumab	KEYNOTE-059 (NCT02335411)	II	316	Cis+5-FU	May-19	11.60%	5.6	2	Rec/Met GC	[37]
			KEYNOTE-061 (NCT02370498)	III	592	Paclitaxel	Jul-19		Failed		Adv GC	[38]
			KEYNOTE-062 (NCT02494583)	III	764	Cis+5-FU(X)	Jun-20				Adv GC	
			KEYNOTE-585 (NCT03221426)	III	860	Cis+X(5-FU) or FLOT	Jul-23				GC	
		Nivolumab +/-Ipilimumab (CTLA4 inhibitor)	ONO-4538-38 (NCT03006705)	III	700	S-1 or XOX	Jun-10				Stage III GC	
			CheckMate649 (NCT02872116)	III	1266	XOX or FOLFOX	Oct-21				Adv/Met GC	
			ONO-4538-37 (NCT02746796)	II/III	680	SOX or XOX	Aug-20				Adv/Rec GC	
			ONO-4538-12 (NCT02267343)	III	480	Nivolumab *vs* Placebo	Aug-17	11.20%	5.32/4.14 *P* < 0.0001	1.61/1.45	Adv/Rec GC	[40]
	PD-L1	Avelumab	JAVELIN Gastric 300 (NCT02625623)	III	371	Avelumab + BSC *vs* Irinotecan + Paclitaxel	Sep-20				Rec/Met GC	
			JAVELIN Gastric 100 (NCT02625610)	III	466	*vs* OX + 5-FU(X)(LV)	Mar 202 4				Adv/Met GC	
		Durvalumab	NCT02572687	I	114	Ramucirumab	Sep-18	36%		2.6	Adv/Met GC	[43,45]
			NCT02340975	Ib/II	135	+/- Tremelimumab (CTLA4 inhibitor)	Aug-19				Rec/Met GC	[46]
			NCT02678182	II	770	Cis, X	Aug-10				Adv/Met HER2 neg. GC	
		Atezolizumab	DANTE (NCT03421288)	II	295	FLOT	Feb-25				Adv GC	
			ICONIC (NCT03399071)	II	40	FLOT-A	Aug-25				T1-T3 GC	
	PIK3CA	BYL719	NCT01613950	I	18	AUY922	Mar-14	NA			Adv/Met GC	
		AZD5363	NCT02451956	II	25	Paclitaxel	Dec-18				Adv GC with PIK3CA mutation	
	ARID1A	PLX2853	NCT03297424	II	166		May-21				ARID1A mutations	
		AZD2281 (Olaparib)	NCT02576444	II	64		Sep-18				PIK3CA, AKT, or ARID1A mutations	
MSI	PD-1	Pembrolizumab	KEYNOTE-016 (NCT01876511)	II	171		Jun-21	40%	Not reached	5.4	MSI	[47]
GS	CDH1	NA	-			Prophylactic gastrectomy						
	RHOA	NA				NA						
	CLDN18-ARHGAP fusion	NA				NA						
CIN	EGFR	Cetuximab	NCT00183898	II	75	XOX	Jun-18				Adv GC	
		Panitumumab	NEOPECX (NCT01234324)	II	171	ECX, placebo	Aug-17				Adv GC incl. GEJ	
			MEGA (NCT01443065)	II	162	FOLFOX/FOLFOX + panitumumab/FOLFOX + AMG102	Jan-19		13.1/8.3/11.5	5.8/5.2/7.6	Adv GC	[48]
		Nimotuzumab	NCT01813253	III	400	Irinotecan, placebo	Jan-18				EGFR overexpr. Adv GC or GEJ	
			NIEGA (NCT03400592)	II	55	Irinotecan	Jun-18				Rec/Met GC with overexpr. EGFR	

HER2	Trastuzumab	NCT01260194	IV	4		Jan-15				GC	
		HELOISE (NCT01450696)	IIIb	248	Cis + X + Herceptin (6 mg/kg or 10 mg/kg)	Aug-15		12.5/10.6 *P = 0.2401*		GC	[49]
		PETRARCA/FLOT6) (NCT02581462)	II/III	404	FLOT or FLOT + Herceptin/ Pertuzumab	Mar-21				HER2+ GC or GEJ	
		NCT02954536	II	37	Pembrolizumab or Pembrolizumab + X/Cis	Nov-19				HER2+ GC	
		Her + XELOX (NCT01396707)	II	55	Herceptin + XELOX	Mar-18		21	9.8	Met/Rec HER2 + GC	[50]
		EVIDENCE (NCT01839500)		95		Feb-18		30	9.5	GC	[51]
	Trastuzumab emtansine	GATSBY (NCT01641939)	II/III	415	or Standard Taxane Therapy	Apr-16		7.9/8.6 *P = 0.8589*	2.66/2.89	Prev. treated for HER2+ Adv GC	[52]
	Pertuzumab	JACOB trial (NCT01774786)	III	780	Trastuzumab + 5-FU/X/Cis, placebo	Dec-21		17.5/14.2 *P = 0.0565*	8.5/7.0	HER2+ Met GC and GEJ	[53]
		NCT01461057	II	30	X + Cis + Trastuzumab	Sep-17				Met HER2 + GC or GEJ	[54]
		INNOVATION (NCT02205047)	II	220	Cis/X or Cis/5-FU +/- trastuzumab, placebo	Sep-24				GC, EGFR overexpress.	[55]
	Margetuximab	NCT02689284	Ib/II	72	Pembrolizumab	Mar-20				Adv/Met HER2 + GC or GEJ	[56]
		NCT01148849	I	67		Dec-17				HER2+ GC	[57]
EGFR/ HER2	Lapatinib	LOGiC/TRIO-013 (NCT00680901)	III	545	XOX, Placebo	Dec-19		11.9/10.4; *P = 0.3244*	6.0/5.4; *P = 0.0381*	GC	[58,59]
		NCT02015169	II	32	XELOX	Nov-17				HER2 + GC with liver metastasis	
VEGF	Bevacizumab (Avastin)	NCT01471470	II	31	Docetaxel + X + Cis	Dec-19		38.6	13.1	Adv GC	[60]
		AGMT_GASTRIC-3 (NCT00952003)	II	40	1 OX + Irinotecan + Avastin; 2 Docetaxel + Avastin; 3 Avastin	Apr-18		11		GC	[61]
		NCT00911820	II	88	Cis+Irinotecan or Docetaxel + Cis + Irinotecan	Jun-18	0.57/0.51; *P = 0.605*	11.7/13.4; *P = 0.714*	7.9/8.4; *P = 0.721*	Met GC	
		NCT01191697	II	35	XOX + trastuzumab	Apr-17		26.92	13.93	HER2 + Met GC	[62]
VEGFR2	Ramucirumab (IMC-1211B)	Rainbow trial (NCT01170663)	III	665	Paclitaxel Placebo	Feb-17		9.6/7.4; *P = 0.0169*	4.4/2.9; *P < 0.0001*	GC	[63,64]
		ARMANI (NCT02934464)	III	280	Ramucirumab + Paclitaxel or FOLFOX4/mFOLFOX6/ XELOX	Oct-19				Adv/ Met HER2- GC or GEJ	
		RAINFALL (NCT02314117)	III	128	X + Cis, Placebo	May-18				Met GC	[65]
		NCT02898077	III	450	Paclitaxel, Placebo	Mar-21				GC	
		RAMSES/FLOT7 (NCT02661971)	II/III	908	FLOT, Placebo	Oct-19				GC or GEJ	
VEGFR2/ TIE2	Regorafenib	INTEGRATE II (NCT02773524)	III	350	Placebo	Apr-19				GC	[66]
		NCT01913639	II	36	FOLFOX, Placebo	Jul-18				GC	[67]

mTOR	Everolimus (RAD001)	AIO-STO-0111 (NCT01248403)	III	300	Paclitaxel, Placebo	Jul-17	8.0%/7.3%; *P* = 0.4	6.1/5.0; *P* = 0.54	2.2/2.07; *P* = 0.3	GC	[68]
	AZD2014	NCT03082833	II	25		Feb-19				TSC1/2 mut. or null GC	
MET	Onartuzumab	NCT01662869	III	562	mFOLFOX6, Placebo	Dec-15		11.0/11.3	6.7/6.8	Met HER2-/+ GC or GEJ	[69,70]

ESCD: Estimated study completion date; ORR: Overall response rate; mOS: Median overall survival (months); mPFS: Median progression free survival (months); Adv/Rec/Met: Advanced/recurrent/metastatic; GEJ: Gastroesophageal junction adenocarcinoma; Cis: Cisplatin; 5-FU: 5'Fluorouracil; S-1: Tegafur-gimeracil-oteracil potassium; X: Capecitabine; OX: Oxaliplatin; XOX: Capecitabine + oxaliplatin; FOLFOX: Leucovorin/fluorouracil/oxaliplatin; SOX: Tegafur/gimeracil/oteracil potassium + oxaliplatin; FLOT: Docetaxel + oxaliplatin + 5FU + leucovorin (calcium folinate); LV: Leucovorin; BSC: Best supportive care; AUY922: Hsp90 inhibitor; ECX: Epirubicin + cisplatin + capecitabine; NA: Not available.

frequency[28]. Most of these aberrant expressed proteins involved in signaling pathways are investigated as possible therapeutic targets for GC anti-tumour therapy and are currently being tested in clinical trials[21,29].

CLINICAL IMPLICATIONS OF MOLECULAR GC CLASSIFICATION: IMPACT ON THERAPY AND PROGNOSIS

The novel classification system, based on the molecular characteristics, have allowed the identification of pathways that contribute to carcinogenesis and underline several driver genes relevant for each GC subtype, that can be used as potential therapeutic targets. In Table 1, the most advanced clinical trials of novel therapeutic strategies are presented, divided for each GC subgroup and molecular target[30-69].

EBV and MSS/TP53+ subtypes

The EBV and MSS/TP53+ subtypes present similar features such as EBV infection, PD-L1/PD-L2, JAK2 overexpression, non-silent mutations in PIK3CA, ARID1A, BCOR and high prevalence of cyclin-dependent kinase inhibitor 2A (CDKN2A) promoter methylation[1,19]. These data suggest that EBV-positive tumors could be targeted with PI3-kinase, JAK2 inhibitors or even better with a combination of those two types of inhibitors which proved strong synergistic effect in other systems[30]. Using checkpoint inhibitors is another therapeutic strategy that is currently evaluated in a series of clinical trials in patients with gastric cancer.

This GC subtype is particularly associated with an enhanced survival due to a strong inflammatory response induced by a major CD8+ cytotoxic T-cell infiltrate as a result of EBV infection. Several studies have shown that EBV can directly increase PD-L1 promoter activity as a result of EBV latent membrane protein 1 (LMP1) binding to JAK3, *via* STAT signaling and AP-1 activation[31,32]. The immune checkpoint ligands PD-L1/PD-L2 overexpression can be a valuable way to block the PD-1 pathway, attenuating the immune response and therefore helping the EBV+ GC to evade immune attack. Additional, IFN-γ released by tumour infiltrating T cells as a result of EBV infection, can directly induce PD-L1 expression in tumour cells[33,34]. Together, these data suggest that EBV+ GC may benefit from anti-PD-1 directed therapy.

Currently there are several PD-1/PD-L1 inhibitors approved by FDA for cancers like non-small-cell lung carcinoma (NSCLC) and melanoma, which are tested for efficacy in ongoing clinical trials on GC. The most known PD-1 inhibitors are: Pembrolizumab (Keytruda®) and Nivolumab (Opdivo®). Additional, PD-L1 approved inhibitors are: Avelumab (Bavencio®), Durvalumab (Imfinzi®) and Atezolizumab (Tecentriq®).

Pembrolizumab is a humanized monoclonal IgG4 antibody directed against human cell surface receptor PD-1 (programmed death-1 or programmed cell death-1) with potential immune checkpoint inhibitory and antineoplastic activities. At this time there are 29 ongoing studies involving pembrolizumab treatment in GC: 13 in phase I, 12 in phase II and 4 in phase III. Based on previous encouraging results obtained by two phase Ib clinical trials: KEYNOTE-012 (NCT01848834) and KEYNOTE-028 (NCT02054806)[35] that evaluated the efficacy of pembrolizumab as single-agent in patients with solid cancers, with a partial response rate of 22%-30%, ongoing efforts are testing pembrolizumab combined with cytotoxic chemotherapy in an attempt to improve the response rate. The phase II KEYNOTE-059 study (NCT02335411) is testing the efficacy of pembrolizumab associated with cisplatin and 5-fluorouracil. Full data from the KEYNOTE-059 trial were presented at the 2017 ASCO Annual Meeting[36]. The

general overall response rate (ORR) with pembrolizumab was 11.6%, and higher in patients who specifically received 2 prior lines of therapy (16.4%). The median PFS was 2.0 mo and the median overall survival (OS) was 5.6 mo, with a 12 mo OS rate of 23.4%. Based on these results, the FDA accelerated the approval of pembrolizumab in September 2017 for the treatment of patients with PD-L1-positive recurrent or advanced GC who has received 2 or more lines of chemotherapy. The accelerated approval of pembrolizumab for this indication was contingent on the results of a confirmatory trial. The ongoing phase III KEYNOTE-062 (NCT02494583) trial is evaluating pembrolizumab alone and in combination with Cis and Capecitabine (5-FU) as the first line therapy for PD-L1-positive GC. KEYNOTE-585 (NCT03221426), another phase III trial, is studying the combination of pembrolizumab and chemotherapy (Cis + Capecitabine (5-FU) or FLOT (do cetaxel+oxaliplatin+5FU+leucovorin) as neoadjuvant and adjuvant therapy. Recently, results from a different phase III trial KEYNOTE-061 (NCT02370498), testing the association of pembrolizumab and paclitaxel, were announced. On Dec 2017, Merck Company, reveal that pembrolizumab (Keytruda®) did not improve survival as a second-line treatment for PD-L1-positive patients with advanced GC[37].

Nivolumab (ONO-4538/BMS-936558) is another human monoclonal IgG4 antibody which blocks the PD-1 receptor. Currently, there are 17 clinical trials on GC involving nivolumab: 8 in phase I, 6 in phase II and 3 in phase III. The activity and safety of nivolumab was first tested in a phase I/II study CheckMate-032 (NCT01928394), as standalone agent or in combination with ipilimumab, an inhibitor for CTLA4, a protein receptor expressed on T-cells that function as an immune checkpoint. This study reported that ORR was 14% in patients treated with nivolumab alone N3 (nivolumab 3 mg/kg corp), 26% in N1+I3 (nivolumab 1mg/kg corp plus ipilimumab 3 mg/kg corp) cohort, and 10% in N3+I1 (nivolumab 3 mg/kg corp plus ipilimumab 1 mg/kg corp) group. The mOS were 6.9 mo in N1+I3 group, followed by 5.0 mo in N3, and 4.8 mo in N3+I1 groups. However the most active regimenN1+I3 had the highest toxicity (84%), compared with N3 (70%) and N3+I1 (75%). Another important outcome of the study was the evaluation of the response rate for nivolumab alone or in combination with ipilimumab depending on the PD-L1 expression. As a result, the response rate for PD-L1 positive tumors was 27% for N3 alone and 44% for N1+I3. For patients with PD-L1 negative tumors the response rate was 12% for N3 and 21% for N1+I3 regimen[38].

More recently, results from ONO-4538-12 (NCT02267343) study were made available. This was a phase III clinical trial aiming to evaluate the efficacy and safety of nivolumab as rescue treatment after failure of the standard chemotherapy for GC. The results showed that nivolumab was effective with significantly improved OS, PFS and ORR compared to placebo. Median OS was 5.32 mo with nivolumab *vs* 4.14 mo with placebo. The ORR was 11.2% with nivolumab *vs* 0 with placebo ($P < 0.0001$). Median progression-free survival (mPFS) was 1.61 mo with nivolumab *vs* 1.45 mo with placebo[39].

Avelumab is a human monoclonal IgG1 antibody directed against the human PD-L1 protein, with potential immune checkpoint inhibitory and antineoplastic activities. Avelumab was approved by FDA as Bavencio® for metastatic Merkel cell carcinoma (MCC) based on multi-center clinical trial (JAVELIN Merkel 200 trial, NCT02155647) that reported a ORR of 29.5% and a mPFS of 2.6 mo, shoing that avelumab has a manageable safety profile with durable responses[40]. Five clinical trials are currently testing avelumab in GC: 1 in phase I, 2 in phase II, and 2 in phase III. In the phase III clinical trials, the avelumab efficacy as single agent is being compared with different chemotherapeutic regimens: Irinotecan + paclitaxel in the JAVELIN Gastric 300 (NCT02625623), and OX + 5-FU(X) (LV) in JAVELIN Gastric 100 (NCT02625610).

Durvalumab (MEDI4736) is an Fc optimized monoclonal antibody directed against PD-L1. It was accepted by FDA and European Medicines Agency (EMA) as Imfinzi® for the treatment of patients with metastatic urothelial carcinoma and locally-advanced (stage III), unresectable non-small cell lung cancer whose disease has not progressed following platinum-based chemoradiation therapy in a phase III PACIFIC trial[41]. Durvalumab is not currently approved for the treatment of patients with gastric or GEJ adenocarcinoma. There are 4 phase Ib/II studies that are currently enrolling patients for treatment with durvalumab as single agent, or in combination with tremelimumab - a CTLA4 inhibitor (NCT02340975) or ramucirumab - a VEGFR-2 inhibitor (NCT02572687). Preliminary results from the last study, presented at the 2018 Gastrointestinal Cancers Symposium, suggests that blocking VEGFR-2 and the PD-1/PD-L1 pathway induces synergic antitumor effects[42].

Atezolizumab is a monoclonal antibody designed to bind with PD-L1. It was approved by FDA and EMA under the trade name Tecentriq® for the treatment of patients with advanced or metastatic non-small cell lung cancer or metastatic urothelial carcinoma after they have been previously treated with chemotherapy. In the field of gastric cancer, there are three studies (1 in phase I and 2 in phase II) that are currently enrolling patients to evaluate the safety and efficacy of atezolizumab in combination with FLOT chemotherapy *vs* FLOT alone, for operable adenocarcinoma of the stomach, the ICONIC (NCT03399071) and DANTE (NCT03421288) studies.

As an important alternative strategy addressing T cell receptors/Ligands interactions is knocking-out the gene for PD-1 receptor by CRISPR-Cas9 DNA-editing technology that might be more effective than using inhibitors or antibodies against it or against the ligands. It is postulated that editing *PD-1* gene will maintain T cell activity in the presence of the checkpoint ligands,

PD-L1 and PD-L2, however ethical aspects of the in-human use of the technology are still debated[43].

PIK3CA inhibitors: A number of PI3K inhibitors are under clinical investigation by pharmaceutical companies and academic institutions, including the pan-PI3K inhibitor buparlisib (BKM120) and the PI3Kα-selective inhibitor alpelisib (BYL719). Buparlisib and alpelisib are currently in Phase III and Phase II clinical trials, respectively for HER2-negative breast cancer (NCT00863655, NCT01610284), HER2-positive breast cancer (NCT01007942), head and neck squamous cell carcinoma (NCT01737450), non-small cell lung carcinoma (NCT01297491), lymphoma (NCT01719250), glioblastoma multiforme (NCT01934361, NCT01349660), preclinical results and initial clinical findings sowing great promise[70].

In GC filed, clinical trials are currently underway, often with PI3K inhibitors in combination with other drugs. The association between BYL719 and HSP90 inhibitor AUY 922, is tested specific in gastric cancer in NCT01613950 trail, and BKM120 with hedgehog pathway inhibitor LDE 225 in advanced solid tumors through NCT01576666 study. The AKT inhibitor AZD5363 is also being tested in the second line in combination with paclitaxel, in gastric cancer patients with and without PIK3CA mutations or amplifications (NCT02451956, NCT02449655)[71].

ARID1A inhibitors: ARID1A protein is a subunit of the SWI/SNF (BAF) chromatin remodelling complex that regulates gene expression by controlling gene accessibility. ARID1A shows one of the highest mutation rates across different human cancer types[13,72-76].

Currently there are no clinical trials in gastric cancer patients involving ARID1A inhibitors. There are however two clinical trials involving patients with solid tumors harbouring ARID1A mutation. First one, NCT03297424 a phase II trial, is testing the efficacy of PLX2853, an inhibitor of the bromodomain-containing protein 4 (BRD4), in subjects with advanced malignancies and ARID1A mutations. The second one NCT02576444, also a phase II trial, is testing patients with solid tumors harbouring PIK3CA, AKT, or ARID1A mutations for response at Olaparib (AZD2281) a poly ADP ribose polymerase (PARP) inhibitor. Both studies are ongoing.

MSI subtype

Due to MMR mechanism deficiency, the MSI subtype has the highest rate of mutations compared to the other groups. A study published by An *et al*[77] demonstrated that patients with an increased number of mutations in MMR genes do not respond to 5-FU chemotherapy. Consequently, chemotherapy may be beneficial only for MSI patients with low mutational burden. This situation is similar to that of MSI colon cancer[78]. Based on the positive results recorded in the treatment of patients with PD-1-positive MSI colon cancer[79], a similar therapy with pembrolizumab was attempted in patients with GC MSI high. Recent results of a Phase II clinical trial KEYNOTE-016 (NCT01876511) showed that this treatment was beneficial[46]. The results can be explained by the findings of a recent study by Cho *et al*[80], which reported an overexpression of PD-L1 in 61.5% of MSI-GC samples that is associated with long-term survival of the patients. Based on this finding, we can conclude that MSI+ status is associated with good prognosis and may be a candidate for immune checkpoint inhibitors therapy due to the sustained response. These results, together with other outcomes from four clinical trials KEYNOTE-164 (NCT02460198), KEYNOTE-012 (NCT01848834), KEYNOTE-028 (NCT02054806), and KEYNOTE-158 (NCT02628067), determined in May 2017 the FDA to grant accelerated approval to pembrolizumab (Keytruda) for patients with solid tumors that have the microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR)[81].

Genomic stable or MSS/EMT subtype

This group is best described as diffuse type of GC, with a low number of mutations that are developed of early age. This form of cancer includes early-onset gastric carcinoma which due its association with some early triggers that impair genome stability, could be considered the best model of gastric cancerogenesis and the best model for testing new treatment options, in the pressing need for new therapeutic for diffuse GC[82]. Another One key finding was that the MSS/EMT subtype showed a higher recurrence rate with peritoneal seeding, and very poor survival compared to other subtypes. EMT is an important mechanism in tissue fibrosis that is characterized by the loss of cell-cell adhesion. Various studies have indicated transforming growth factor (TGF)-b1, secreted by gastric cancer cells and cancer-associated fibroblasts (CAFs), as common initiator of EMT[83,84]. Saito *et al*[85] successfully used tranilast, an inhibitor of TGF-b/Smad pathway to inhibit interactions between cancer cells and stroma, preventing fibrous tumor establishment represented by peritoneal dissemination.

Currently effective targeted therapies for diffuse GC are lacking. A significant key in our understanding of diffuse GC was the discovery of hereditary form carried out by missense mutation in CDH1 gene, which encodes E-cadherin. Additional, novel recurrent mutations of RHOA and CLDN18-ARHGAP6 fusion were identified and linked with diffuse GC pathogenesis.

CDH1: Currently there are only three clinical trials regarding CDH1 in gastric cancer: Two of them are observational, aiming to determine the incidence of CDH1 germline mutations among individuals with early onset or familial gastric cancer and their relatives (NCT00582257, NCT03030404), and third one is prospective, aiming to investigate the association between the level of CDH1 methylation (among other genes) and metastasis/

recurrence of gastric carcinomas (NCT02159339). At the moment, the primary clinical approach is curative prophylactic gastrectomy at early age, since the lifetime risk of developing GC for CDH1 mutation carriers with familial history of hereditary diffuse gastric cancer is 60%-80%, with a mean age of onset of 37 years[86].

RHOA: RHOA mutations were found in 14%-25% of diffuse GC patients[87,88]. RHOA, as a member of the Ras GTPase superfamily, is a critical transducer of extracellular signals, which leads to organization of actin cytoskeleton, motility, adhesion and gene regulation. Overexpression of RHOA or constitutive activation was observed in various cancers, and was associated with implicated in tumorigenesis and tumor cell invasion[89]. Also, several mutations were found in diffuse GC with tumor promoting activity, as demonstrated by small interfering RNA (siRNA)-mediated silencing studies[90]. There are no currently available clinical trials or directed treatment for RHOA mutations. A study performed by Kang *et al*[91]. aimed to explore whether RHOA influences the susceptibility of gastric cancer cells to chemotherapeutic drugs founded that RHOA significantly enhanced the resistance to lovastatin, 5-FU, taxol and vincristine, but did not affect the sensitivity to cisplatin or etoposide in SNU638 human carcinoma cell line.

CLDN18-ARHGAP6 or 26 fusions are another genomic alterations associated with GS type of GC. It is mutually exclusive with RHOA and CDH1 mutations among GS tumors[92]. ARHGAP 26 gene is encoding a GTP-ase protein that modulates RHOA activation. Moreover, this genomic fusion may affect CLDN18 function, a protein involved in cell adhesion.

Currently, there are no targeted therapies for the gene mutations and alterations involved in this GC subtype. However, these known mutations may facilitate the development of novel therapies.

CIN and MSS/TP53-subtypes

Characterization of GC at the molecular level allowed also the identification of the intracellular pathways that might contribute to carcinogenesis. Studies to date point out that several signaling cascades are implicated in gastric carcinogenesis including: ERBB, VEGF, PI3K/AKT/mTOR, and HGF/MET signaling pathways. There are currently numerous studies using inhibitors of ERBB, MET, PI3K, AKT or mTOR signaling molecules, but often attempting to block one these paths leads to activation of other cascade due to compensatory signaling[93]. However, Trastuzumab and Ramucirumab (targeting HER2 and VEGFR2 respectively) are the only targeted therapies approved so far, and most Phase III clinical trials evaluating molecular drugs targeting signaling molecules in gastric cancer have failed[94].

ERBB signaling pathways: ERBB receptors (1-4) are members of the RTK superfamily, localized to the cell surface, with high-affinity for many growth factors, cytokines, and hormones. These molecules have a predominantly regulatory role in nearly every aspect of cell biology and also a critical role in the development and progression of many types of cancer, including gastric cancer. Activation of ERBB1 (EGFR) and ERBB2 (HER2) due to amplification or mutations has been reported in almost 30% of gastric cancer patients and overexpression of these receptors has been associated with advanced stages of gastric cancer, more aggressive disease and a poor prognosis[95-97].

Several strategies for the targeting of EGFR signaling using small molecules tyrosine kinase inhibitors and monoclonal antibodies have been developed and used in preclinical and early phase clinical studies, like Cetuximab, Panitumumab, Trastuzumab, Pertuzumab, and also EGFR tyrosine kinase inhibitors such as Lapatinib[98].

Cetuximab is a chimeric human-murine IgG1 monoclonal antibody which binds to the extracellular domain of EGFR leading to the internalization of antibody-receptor complex and downregulation of EGFR expression. Cetuximab-EGFR complex formation inhibits proliferation and enhances apoptosis, as well as reduces angiogenesis, invasiveness and metastasis in tumours[99]. Also, cetuximab induces antibody-dependent cellular cytotoxicity (ADCC)[100] through NK and CD8+ T cells[101]. Cetuximab is approved by FDA[102] and EMA[103] as Erbitux® for the treatment of K-ras wild-type, EGFR-expressing metastatic colorectal cancer and for squamous cell carcinoma of the head and neck (SCCHN). Regarding GC, cetuximab is reported, alone or in combination with other drugs, in 20 clinical trials on clinicaltrials.gov: 1 in phase III, 14 in phase II, 2 in phase I/II, and three in phase I. In the phase II EXTRA trial (NCT00477711), 47 patients were enrolled with unresectable or metastatic GC from China. After administration of cisplatin, capecitabine and cetuximab, the ORR was 53.2%, mPFS 5.2 mo, and OS 10.8 mo. In the case of patients with EGFR strong expression longer PFS (7.1 mo) and OS (16.6 mo) were registered, as well as tumour reduction. Moreover, patients with high levels of transforming growth factor-alpha (TGF-α) presented better response and longer PFS (6.0 mo *vs* 2.7 mo, P = 0.001) and OS (12.9 mo *vs* 7.0 mo, P = 0.001) compared to patients with lower levels[104]. Still, comparing the administration of cetuximab with capecitabine and cisplatin and of capecitabine and cisplatin alone in EXPAND trial (NCT00678535), the addition of cetuximab to first-line chemotherapy provided no additional benefit in the treatment of GC. EXPAND is a phase III trial financed by Merck KGaA, in which were enrolled 904 patients with histologically confirmed locally advanced unresectable or metastatic GC and tested regimens were capecitabine and cisplatin with or without cetuximab. The progression-free survival (PFS) of patients receiving cetuximab comparing with those receiving only first-line chemotherapy was 4.4 mo *vs* 5.6 mo (P = 0.32), while overall survival (OS) was 9.4 mo *vs* 10.7 mo (P

= 0.9547). Moreover, 54% of patients in the cetuximab group had any grade of serious adverse event *vs* 44% in the control group[105]. At present, cetuximab is implicated in several ongoing trials on GC involving NK cells (NCT03319459) or combinations with other monoclonal antibodies (NCT02318901).

Panitumumab is a human IgG2 monoclonal antibody that binds to EGFR and, similar to cetuximab, induce its internalization leading to increased apoptosis, reduced proliferation and reduced angiogenesis and metastasis[106,107]. Panitumumab is approved by FDA[108] and EMA[109] as Vectibix™ for treatment of colorectal cancer. In GC, panitumumab was tested in several clinical trials. In the open-label phase III REAL trial (NCT00824785) were enrolled 553 patients with untreated, metastatic, or locally advanced GC and a combination of epirubicin, oxaliplatin, and capecitabine (EOC) with/without panitumumab was tested. OS in patients that received EOC plus panitumumab was 8.8 mo *vs* 11.3 mo in patients that were allocated only EOC (P = 0.013). Moreover, EOC plus panitumumab induced more severe adverse effect. The study was interrupted in 2011 by independent data monitoring committee due to lack of efficacy, the addition of panitumumab to EOC chemotherapy being not recommended for use in an unselected population[110].

In phase II study (VEGA trial) were included 65 patients with metastatic GC. After allocation of oxaliplatin, leucovorin, and 5-fluorouracil (FOLFOX) plus panitumumab, registered ORR was 42%, PFS-5.6 mo, and mOS-11 mo[111].

The phase II study, reported by Tomasello *et al*[112], explored panitumumab in combination with docetaxel, cisplatin, folinic acid and 5-fluorouracil (DCF) administrated to 52 patients with HER2 negative locally advanced or metastatic GC. The registered ORR was 64% and mOS was 10 mo, still, the toxicity profile of this regimen limits its further development[113]. At present, on clinicaltrials.gov, panitumumab is used in 4 clinical trials on GC: 1 in phase III, 2 in phase II, and 1 in phase I/II from which one is ongoing.

Nimotuzumab (h-R3) is a humanized IgG1 monoclonal antibody[114] that binds to the extracellular domain of EGFR and inhibits EGFR-dependent transformation of cells proving also anti-proliferative, pro-apoptotic, and antiangiogenic activity[63]. Although nimotuzumab is less toxic than other anti-EGFR antibodies[64], it is still not approved by FDA or EMA. At present, in GC, nimotuzumab is reported in four clinical trials on clinicaltrials.gov: 1 in phase III and 3e in phase II. In a phase II randomized trial (NCT02370849) were enrolled 62 patients with untreated unresectable or metastatic GC which received S-1 and cisplatin with or without nimotuzumab. The ORR was 54.8% for patients receiving S-1, cisplatin, and nimotuzumab compared with 58.1% for those receiving S-1 and cisplatin alone (P = 0.798), mPFS were 4.8 mo *vs* 7.2 mo (P = 0.011), while OS were 10.2 mo *vs* 14.3 mo (P = 0.062). The addition of nimotuzumab to S-1 and cisplatin regimen offered no additional benefit in the first-line treatment of GC[115]. Also, nimotuzumab proved no advantage in combination with irinotecan (a phase II study on 82 irinotecan-naive patients with Adv GC), with registered PFS of 73.0 d *vs* 85.0 d (P = 0.5668), and mOS of 250.5 d *vs* 232.0 d (P = 0.9778) for the patients receiving irinotecan with nimotuzumab *vs* those receiving irinotecan alone. Still, this combination presented a potential improvement in the EGFR 2+/3+ group (PFS: 118.5 d *vs* 59.0 d and OS: 358.5 d *vs* 229.5 d)[116].

Trastuzumab is a humanised IgG1 monoclonal antibody that targets the extracellular domain of HER2 and prevents HER2 shedding, inhibits MAPK and PI3K/Akt pathways, and induces ADCC by activating NK cells[117,118]. Trastuzumab is approved by FDA[119] and by EMA[120] for treatment of breast cancer and of metastatic GC. At present, on clinicaltrials.gov, there are 67 studies on GC including trastuzumab: 2 in phase IV, 5 in phase III, 2 in phase II/III, 29 in phase II, 8 in phase I/II (including 2 ongoing studies that imply NK cells) and 10 studies in phase I. In ToGA study (phase III, NCT01041404), trastuzumab administrated with fluoropyrimidine/cisplatin improved PFS (6.7 *vs* 5.5, P = 0.0002) and mOS (13.8 *vs* 11.1, P = 0.0046) in patients with HER2+ advanced GC[121]. In a phase II study including patients with HER2+ advanced GC (NCT01228045), the use of trastuzumab with S-1 and cisplatin induced mOS of 14.6 mo and mPFS of 7.4 mo, at 6 mo 62.6% of patients being free from disease progression, this combination proving good activity and being well tolerated[122]. The combination of trastuzumab with oxaliplatin/capecitabine in patients with HER2+ advanced GC was evaluated in other phase II clinical trial (CGOG1001, NCT01364493). The registered PFS and mOS were 9.2 and 19.5 mo, respectively. Patients with a greater HER2/CEP17 ratio proved a better OS (20.9 mo *vs* 19.5 mo, P = 0.001)[123].

In a retrospective study reported by Palle *et al*[124] were evaluated 104 patients with HER2 + GC who received after first-line chemotherapy (fluoropyrimidine and cisplatin or oxaliplatin, plus trastuzumab), one of the following second line chemotherapy regimens: FOLFIRI, taxane (paclitaxel or docetaxel) or platinum-based chemotherapy (different from that used in first-line), with or without trastuzumab. The continuation of trastuzumab was associated with an increased PFS (4.4 mo *vs* 2.3 mo, P = 0.002) and OS (12.6 mo *vs* 6.1 mo, P = 0.001). Therefore the administration of trastuzumab in combination with second line chemotherapy proves clinical benefit in patients with HER2+ advanced GC[124].

Pertuzumab is a humanized monoclonal antibody that inhibits HER2 by preventing its dimerization and acts at different epitope than trastuzumab[125]. Pertuzumab is approved by FDA[126] and by EMA[127] for treatment of HER2-positive breast cancer. Regarding gastric cancer pertuzumab is implicated in 5 clinical trials (4 ongoing) reported on clinicaltrials.gov: one in phase III, one in phase II/III and 3 in phase II, but still proved no benefit, alone or in combination with other drugs.

Lapatinib is a tyrosine kinase inhibitor that inhibits both HER2 and EGFR[128]. It is approved by FDA as Tykerb®[129] and by EMA as Tyverb[130] for breast cancer treatment. In gastric cancer, lapatinib is included in 10 clinical trials on clinicaltrials.gov: 2 in phase III, 6 in phase II and 2 in phase I. In the phase III TyTAN trial (NCT00486954), including Asian patients with Her2+ GC, mOS/PFS were 11.0/5.4 mo in patients receiving lapatinib plus paclitaxel *vs* 8.9/4.4 mo in patients receiving paclitaxel alone (P = 0.1044), the combination of lapatinib and paclitaxel proving activity in the second-line treatment of patients with HER2+ advanced GC[131].

VEGF signaling pathway: VEGF signaling represents an essential factor for tumour angiogenesis and lymphangiogenesis being involved in growth, invasion, and metastatic spread of solid neoplasms. VEGF family consists of 7 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF (placental growth factor), which act through specific tyrosine kinase receptors (VEGF VEGFR1, VEGFR2, and VEGFR3). VEGF expression and serum levels are correlated with advanced stage disease and poor outcome in gastric cancer patients. Preclinical studies showed that EGFR2 inhibition decreased tumor growth and angiogenesis[132,133]. The most important approaches for targeting VEGF signaling use the monoclonal antibodies Bevacizumab (anti-VEGF-A mAb), and Ramucirumab (anti-VEGFR-2 mAb) and also VEGFR-targeted tyrosine kinase inhibitors: Sunitinib, Sorafenib, Cediranib, and Apatinib[134].

Bevacizumab (Avastin) is a humanized monoclonal antibody that targets VEGF proving an anti-angiogenic effect[135]. It is approved by FDA[136] and EMA[137] for treatment of metastatic cancer of the colon (large intestine) or rectum, metastatic breast cancer, advanced non-small cell lung cancer, advanced or metastatic kidney cancer, epithelial cancer of the ovary, cancer of the fallopian tube or the peritoneum) and persistent, recurrent or metastatic cervix cancer. Regarding GC, bevacizumab is implicated in 23 clinical trials reported on clinicaltrials.gov: 1 in phase IV (status unknown), 2 in phase III, 16 in phase II, 2 in phase I/II and 2 in phase I. In the phase III AVAGAST trial (NCT00548548) the addition of bevacizumab to capecitabine, 5-fluorouracil and cisplatin regiment improved PFS (6.7 mo *vs* 5.3 mo) and mOS (12.1 mo *vs* 10.1 mo) of patients with locally advanced or metastatic GC. The obtained results highlighted the influence of regional healthcare environment on overall survival differences observed in this study[138]. Based on these conclusions, in the phase III AVATAR study (NCT00887822), bevacizumab was tested in combination with capecitabine and cisplatin in Chinese patients with advanced gastric cancer. PFS and OS were similar in both tested arms (with/without bevacizumab): 6.3 *vs* 6.1 (P = 0.3685) and 10.5 *vs* 11.4 (P = 0.8636) respectively, and no new safety signals were reported comparing with AVAGAST study[139]. In a phase II study reported by Kim *et al*[60], bevazicumab was tested in combination with docetaxel, capecitabine, and cisplatin on patients with unresectable locally advanced or metastatic GC. The registered mPFS and OS were 13.1 mo and 38.6 mo, respectively[60]. In GASTRIC-3 trial, the combination of bevacizumab with oxaliplatin and irinotecan followed by docetaxel, followed by bevacizumab maintenance, was tested on patients with advanced GC. PFS was 7.0 mo and mOS was 11 mo. Notable, two patients continued on bevacizumab maintenance for more than 5 years showing long-term tumour remission. As such, the regimen formed of oxaliplatin/irinotecan and docetaxel plus bevacizumab followed by bevacizumab maintenance is feasible for metastatic GC treatment[61].

Ramucirumab is a humanized monoclonal antibody that targets selectively VEGFR2 and inhibits angiogenesis[140]. It is approved by FDA[141] and EMA[142] for advanced GC, metastatic colorectal cancer, and non-small cell lung cancer treatment. The clinical phase III studies REGARD (NCT00917384) (best-supportive care with/without ramucirumab) and RAINBOW (NCT01170663) (paclitaxel with/without ramucirumab) demonstrated significant survival benefits and manageable toxicity of ramucirumab administration in patients with advanced GC[63,64,114,143,144]. Based on the results obtained in RAINBOW trial, Ramucirumab has been approved by FDA as a single agent or in association with paclitaxel for the treatment of the patients with advanced or metastatic gastric and gastroesophageal junction[95]. At present, on clinicaltrials.gov ramucirumab is implicated in 19 studies: 5 in phase III, one in phase II/III, 6 in phase II, 3 in phase I/II, and 4 in phase I.

Apatinib (YN968D1) is a selective inhibitor for VEGFR2, also slightly inhibiting c-Kit and c-Src tyrosine kinases[145]. Currently it is involved in 39 clinical trials on GC according to clinicaltrials.gov: 3 in phase IV, 4 in phase III, 5 in phase II/III, 17 in phase II, 1 in phase I/II and 3 in phase I. In a phase III clinical trial (NCT01512745) involving patients with advanced GC, apatinib showed an improved PFS (2.6 mo *vs* 1.8 mo, P = 0.0156) and mOS (6.5 mo *vs* 4.7 mo, P = 0.0149) comparing with placebo[146,147]. Administration of apatinib induces progression-free survival rather than post-progression survival[148]. In an observational study, apatinib seems to be more effective in patients previously treated with antiangiogenic therapy[149].

In conclusion, considering all the studies presented, it can be ascertain that drugs directed against VEGFR-2 are more effective than those targeting VEGF-A

PI3K/AKT/mTOR signaling pathway: This signaling cascade is frequently perturbed in human cancers being associated with therapy resistance. Mutations and/or amplifications of PIK3CA gene or loss of function of tumour suppressor protein PTEN were identified in GC, especially in EBV and the MSI subtypes[1,150]. However, phase III and II studies evaluating inhibitors of mTOR

(Everolimus) and AKT (MK-2206) respectively, reported negative results. It should be noted that in these studies the patients were not selected based on PI3K signaling pathway activation[151,152]. The AKT inhibitor AZD5363 effects are being evaluated in combination with paclitaxel in patients with advanced gastric adenocarcinoma harboring PIK3CA mutation or amplification as a second line chemotherapy (NCT02451956). As well, AZD2014, an mTOR inhibitor that targets both mTORC1 and mTORC2 complexes[153], is implicated in phase II clinical studies as second-line chemotherapy.

Everolimus (RAD001) is a rapamycin analog that targets mTOR[154]. It is approved by FDA[155] and by EMA[156] as Afinitor® for breast cancer, pancreatic neuroendocrine tumours, neuroendocrine tumors originating in the lungs or gut, and for advanced renal cell carcinoma treatment. In GC everolimus is tested in 14 clinical trials on clinicaltrials.gov: 2 in phase III, 5 in phase II, 3 in phase I/II and 4 in phase I. Everolimus was evaluated in combination with paclitaxel in a phase III trial (NCT01248403) in patients with advanced GC pretreated with fluoropyrimidines-containing regimen, the obtained PFS and mOS in patients that received paclitaxel with everolimus compared to those that received only paclitaxel being 2.2 mo *vs* 2.07 mo (P = 0.3) and 6.1 *vs* 5.1 mo (P = 0.48), respectively[68]. As a result, the addition of everolimus to paclitaxel does not improve the outcomes in pretreated metastatic GC. In other phase III study (GRANiTE-1, NCT00879333), everolimus efficacy and safety was compared with that of best supportive care (BSC) in patients with previously treated advanced GC. PFS were 1.7 mo *vs* 1.4 mo for patients that received everolimus *vs* placebo group, while mOS were 5.4 mo *vs* 4.3 mo (P = 0.124), respectively[151].

HGF/HGFR signaling: Hepatocyte growth factor (HGF) and its receptor encoded by the MET gene, play key roles in tumour growth through activated signaling pathways in GC cells. Genomic amplification of MET was identified in 4% of patients with gastric cancer and is related to survival. Moreover, preclinical studies suggest that inhibition of MET expression using antibodies or small-molecule inhibitors suppress tumor-cell proliferation and tumor progression in MET-amplified GC cells. Several molecules targeting the MET/HGF signaling have been developed, including monoclonal antibodies [against either HGF (Rilotumumab), or MET (Onartuzumab)] and MET small kinase inhibitors (Foretinib). These inhibitors were evaluated in clinical trials as target therapies for metastatic or unresectable gastric cancer[157]. Unfortunately, negative results were obtained in several phases II and III clinical trials due to a higher rate of serious toxicities or an increase in the number of deaths[158]. The failure of these trials could be explained by an inappropriate selection of the patients that could benefit from the therapy, emphasizing the need to identify new inhibitors for this molecule.

Onartuzumab is an *E. coli*-derived humanized monoclonal antibody that targets MET by binding to the extracellular domain of the receptor and inhibits HGF binding[159]. At the moment it is involved in two clinical trials on GC on clinicaltrials.gov: one in phase III and one in phase II. In the phase II study (NCT01590719) onartuzumab was investigated in combination with mFOLFOX6 in patients with metastatic EGFR2-negative GC. In mFOLFOX6 plus onartuzumab group, the PFS and mOS were 6.77 and 10.61 mo, respectively, while in the mFOLFOX6 plus placebo group, the PFS and mOS were 6.97 (P = 0.71) and 11.27 mo (P = 0.83), respectively. In the MET+ population, PFS were 5.95 mo *vs* 6.80 mo (P = 0.45) and mOS were 8.51 mo *vs* 8.48 mo (P = 0.80) in the onartuzumab *vs* placebo group. Therefore, the addition of onartuzumab to mFOLFOX6 regiment did not improve the outcome of GC patients, including MET+ ones[160].

Rilotumumab (AMG102) is a human IgG2 monoclonal antibody that targets HGF and inhibits HGF/MET signaling[161]. Althought at present on clinicaltrials.gov are mentioned 5 trials on GC that use rilotumumab, only one of them is ongoing - a phase II study that evaluate the combination of FOLFOX with AMG 102 or Panitumumab. Two studies (phase III), RILOMET 1 (NCT01697072) and RILOMET 2 (NCT02137343) were stopped early by Amgen (rilotumumab developer) due to higher number of deaths in the rilotumumab group than in the placebo group[161,162].

Even if there are a lot of clinical trials testing a wide range of inhibitory molecules in GC patients, Trastuzumab and Ramucirumab (targeting HER2 and VEGFR2 respectively) are the only targeted therapies approved so far.

CONCLUSION

Given the large number of clinical trials currently taking place, one may ask why only two compounds have been validated for GC therapy. How can be explain these many failures? First of all, these drawbacks may be due to the high degree of intra-tumour heterogeneity frequently displayed by GC and to the fact that most often clinical trials did not performed a preliminary patient selection for the molecular alteration of the target, a mandatory issue if it is desired to test the efficacy of certain inhibitor to the given target. From this point of view, the recent in-depth molecular studies that allowed stratification of patients in GC subtypes based on genomic alteration, represent a substantial advance as well as a powerful tool for targeting therapy. The implementation of the novel system in which GC patients can be classified in molecular subtypes will bring a real progress in clinical trials, allowing the evaluation of the efficiency of each compound in specific molecular subtypes. Moreover, the new perspective opened by the successful use of immune checkpoints inhibitors in PD-1 and PD-L1/2 positive GC like EBV positive, MSS/TP53+ or MSI subtypes, can open the way for combination of emerging immunotherapies with molecularly targeted drugs, and these findings could

have important translational relevance in the field of GC therapy. Finally, we can conclude that in the attempt to improve the quality of medical care, we have to increase the speed in GC research fields and to develop new therapeutic approaches with high clinical benefits and minimum adverse effects.

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REVIEW

Ambiguous roles of innate lymphoid cells in chronic development of liver diseases

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Abstract

Innate lymphoid cells (ILCs) are defined as a distinct arm of innate immunity. According to their profile of secreted cytokines and lineage-specific transcriptional factors, ILCs can be categorized into the following three groups: group 1 ILCs (including natural killer (NK) cells and ILC1s) are dependent on T-bet and can produce interferon-γ; group 2 ILCs (ILC2s) are dependent on GATA3 and can produce type 2 cytokines, including interleukin (IL)-5 and IL-13; and, group 3 ILCs (including lymphoid tissue-like cells and ILC3s) are dependent on RORγt and can produce IL-22 and IL-17. Collaborative with adaptive immunity, ILCs are highly reactive innate effectors that promptly orchestrate immunity, inflammation and tissue repair. Dysregulation of ILCs might result in inflammatory disorders. Evidence regarding the function of intrahepatic ILCs is emerging from longitudinal studies of inflammatory liver diseases wherein they exert both physiological and pathological functions, including immune homeostasis, defenses and surveillance. Their overall effect on the liver depends on the balance of their proinflammatory and antiinflammatory populations, specific microenvironment and stages of immune responses. Here, we review the current data about ILCs in chronic liver disease progression, to reveal their roles in different stages as well as to discuss their therapeutic potency as intervention targets.

Key words: Innate lymphoid cells; Chronic liver disease; Hepatitis; Liver fibrosis; Liver cancer

Core tip: Innate lymphoid cells (ILCs), mirroring both the phenotypes and functions of T cells, have been defined as a distinct arm of innate immunity. There has been a marked increase in the studies investigating the dysregulation of ILCs in chronic liver pathologies. This manuscript presents a comprehensive overview of the state of ILCs, including the fundamental concepts as well as summarizing their ambiguous roles in the progression of the chronic liver hepatitis, fibrosis and carcinoma. It also provides an insight into the current research gaps and indicates the therapeutic potency and development direction of future research of ILCs.

Shen Y, Li J, Wang SQ, Jiang W. Ambiguous roles of innate lymphoid cells in chronic development of liver diseases. *World J Gastroenterol* 2018; 24(18): 1962-1977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1962.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1962

INTRODUCTION

Liver diseases usually evolve from inflammation to fibrosis, with cirrhosis manifested in the advanced stage, and serving as a well-determined major risk factor of liver cancer. Liver disease remains a major health problem, affecting millions of people worldwide. Ongoing chronic inflammation in the liver induced by infections, hepatotoxic drugs, autoimmunity, alcohol abuse or toxins will result in liver fibrosis, which is the consequence of an irreversible, progressive condition occurring in most types of chronic liver diseases and characterized by excessive deposition of extracellular matrix (ECM) proteins, mainly composed of collagen[1]. The situation where ECM formation is prompted by activated hepatic stellate cells (HSCs) outweighs the collagen degradation by matrix metalloproteases (MMPs) and will lead to structural distortion of the normal liver tissue and functional impairment; furthermore, it is associated with an increased risk of cirrhosis, portal hypertension and subsequent liver failure and liver cancer[2-4]. Tremendous efforts have been made to design strategies which could prevent liver disease progression.

Innate lymphoid cells (ILCs) are a recently identified group of mononuclear hematopoietic cells which encompass not only cytotoxic natural killer (NK) cells but also noncytotoxic ILCs, and are involved in immunity and tissue remodeling. Though characterized with lymphoid morphology, ILCs lack the rearranged antigen receptors and are defined as cell lineage marker-negative (Lin^-) cells[5]. ILCs mirror both the phenotypes and functions of T cells, for which noncytotoxic ILCs have been proposed as the innate counterparts of $CD4^+$ T helper (Th) cells, whereas NK cells are considered to be the innate equivalents of $CD8^+$ cytotoxic T (Tc) cells[6].

Group1 ILCs comprise both Eomes-dependent NK cells and T-bet-dependent ILC1s[7]. Upon stimulation by interleukin (IL)-12, IL-15 and IL-18 derived from both myeloid cells and nonhematopoietic cells, the ILC1s can produce Th1 cell-associated cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which play critical roles in clearing intracellular pathogens[8,9]. Distinguished from ILC1s, NK cells depend on Eomes to develop and exert their cytotoxic functions by secreting granzymes and perforin[10,11]. Group 2 ILCs (ILC2s), being dependent on GATA3 and ROR-α and respondent to epithelium-derived cytokines IL-25, IL-33 and thymic stroma lymphopoietin (TSLP), can produce Th2 effector cytokines (IL-4, IL-5, IL-9, IL-13 and amphiregulin), thus playing a critical role in antihelminth immunity and allergic inflammation as well as tissue repair[12-15]. Finally, group 3 ILCs are dependent on RORγt and mainly respondent to myeloid cell-derived IL-1β and IL-23. These ILCs include lymphoid tissue-like (LTi) cells and ILC3s, which can produce IL-22, IL-17, granulocyte macrophage colony-stimulating factor and TNF, thus showing great significance in antibacterial immunity[16-18] (Figure 1).

Dysregulation of ILCs can cause severe inflammation and injury in gut[19], lung[20], skin[21] and liver[22]. During the past 5 years, a growing number of studies have investigated the roles of ILCs in inflammatory, fibrotic and cancerous liver diseases[23-27]. Herein, we summarize the present knowledge of ILCs to reveal their complicated and versatile effects and the underlying mechanism in chronic liver diseases, in order to provide perspectives of new therapeutic strategies.

LIVER INFLAMMATION

Group 1 ILCs

There are two distinct NK populations in murine liver, $CD49a^-CD49b^+$ and $CD49a^+CD49b^-$ cells. The $CD49a^-CD49b^+$ subset represents conventional (c)NK cells, which circulate in the blood. The $CD49a^+CD49b^-$ subset has ever been considered as tissue-resident (tr)NK cells or ILC1s in previous studies, which are beside dendritic cells (DCs) localizing in the sinusoids of the portal area[28,29]. Both cNK and trNK cells express natural cytotoxicity receptors and require IL-15 signaling for their development. Compared to cNK cells, trNK cells have relatively lower expression of CD11b, Ly49 receptors, CD43 and KLRG1, but higher expression of CXCR3 and CXCR6, which is a chemokine receptor for CXCL16 responsible for the enrichment of natural killer T (NKT) cells in the liver and can provide intravascular immune surveillance[30,31]. In parallel, human livers contain $CD56^{bright}$ and $CD56^{dim}$ (accounting for 90%) NK cells, respectively representing the equivalents of murine cNK and trNK cells. With respect to function, $CD56^{bright}$ NK cells are prominent cytokine producers,

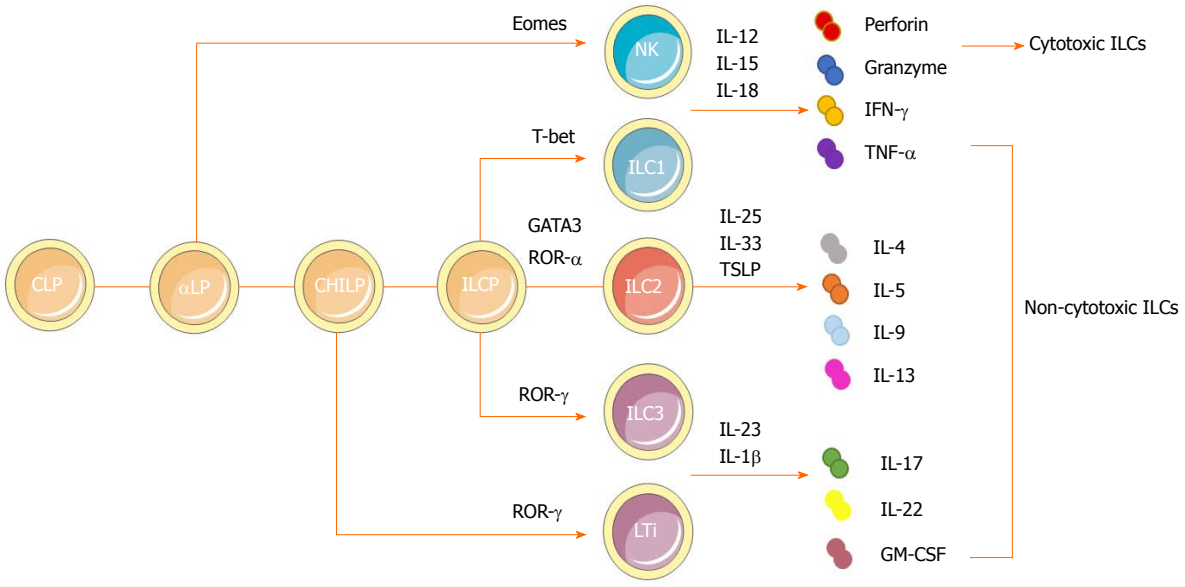

Figure 1 Developmental pathways and classification of innate lymphoid cells. ILCs are derived from a CLP. With the same phenotype as CLP as well as expressing α4β7 integrin, αLP gives rises to cytotoxic NK cells and differentiates into a CHILP, which gives rise to all noncytotoxic ILCs. The transcription factor PLZF further divides the progeny of CHILPs into a PLZF$^+$ ILCPs that are restricted to ILCs except LTi cells and PLZF-independent LTi cells. Group 1 ILCs comprise both Eomes-dependent NK cells and T-bet-dependent ILC1s. They could produce IFN-γ and TNF-α in response to the stimulation by IL-12, IL-15 and IL-18. NK cells can also secrete granzymes and perforin to exert cytotoxic functions. Dependent on GATA3 and ROR-α as well as respondent to cytokines IL-25, IL-33 and TSLP, group 2 ILCs could produce Th2 effector cytokines (IL-4, IL-5, IL-9 and IL-13 and amphiregulin). Group 3 ILCs encompass both RORγ-dependent LTi cells and ILC3s. They can produce IL-22, IL-17 and GM-CSF, mainly in response to IL-1β and IL-23. αLP: α-lymphoid progenitor; CHILP: Common helper-like innate lymphoid progenitor; CLP: Common lymphoid progenitor; GM-CSF: Granulocyte macrophage colony-stimulating factor; IL: Interleukin; ILC: Innate lymphoid cell; ILCP: Innate lymphoid cell precursor; INF: Interferon; LTi: Lymphoid tissue-like; NK: Natural killer; PLZF: Promyeloid leukemia zinc finger; Th: T helper; TNF: Tumor necrosis factor; TSLP: Thymic stroma lymphopoietin.

whereas CD56^{dim} NK cells are efficient killers[32].

Liver is an immune-tolerant organ predisposing for chronic infections of certain clinically significant pathogens. As an organ with predominant innate immunity, the liver is enriched with NK cells, which account for 25%-50% of total intrahepatic lymphocytes and are responsible for killing transformed cells and viruses. The cytotoxicity of NK cells is regulated by both cytokines and surface receptors[33,34]. IFN-γ is one of the most prominent cytokines derived from NK cells to exert antiviral, antifibrotic and antitumorigenic effects. Furthermore, it has been demonstrated that cNK rather than trNK cell-derived IFN-γ promotes Th1 polarization and secondary CD8$^+$ cytotoxic lymphocyte (CTL) responses, the major effectors for clearance of hepatic B virus (HBV) in transgenic HBV mouse models[35]. Meanwhile, the interactions of NK cells with hepatocytes *via* the NKG2A inhibitory receptor could prime DCs to induce CD4$^+$CD25$^+$ regulatory T cells (Tregs), which will in turn up-regulate the expression of NKG2A on NK cells *via* IL-10 production, thus impairing the antiviral ability of NK cells[36,37].

In the pathogenesis of chronic HBV infection (CHB), ILC1s have potential proinflammatory effects that mirror Th1 cells in adaptive immunity exactly. First, in patients with CHB, liver injury has been significantly associated with enhanced ILC1s' response, as reflected by markedly elevated levels of T-bet, IFN-γ and IL-12 signaling. Besides, decreased ILC1-produced IFN-γ has been found to have a connection with the telbivudine-induced alleviation of liver injury in CHB patients[23]. These results could be explained by the study of Krueger *et al*[38], in which it was demonstrated that CD49a$^+$ ILC1s could inhibit expression of CXCL9, which was further required for robust accumulation of IFN-γ$^+$CD49b$^+$ NK cells during the early phase of adenovirus infection. In this way, ILC1s played a role in maintaining the liver as a tolerogenic site as a result of increased expression of NKG2A receptors compared with NK cells, which would further suppress the activation of liver CD103$^+$ DCs, thus interrupting the priming of antigen-specific, antiviral CD8$^+$ T cells and the clearance of virus. The mechanism was found to be the same in hepatitis C virus infection for which *NKG2A*$^{-/-}$ patients showed resistance[39,40]. To conclude, ILC1s help to maintain the tolerance of liver in normal conditions, and blockage of NKG2A signaling to generate potent anti-viral CD8$^+$ T cell responses required for the elimination of persistent liver pathogens may prove to be a novel therapeutic strategy (Figure 2A).

Group 2 ILCs

IL-33 belongs to the IL-1 superfamily, which is alarmins secreted by epithelial cells upon cellular stress and tissue damage. Upon binding to its specific heterodimeric receptor which comprises the ST2 and IL-1 receptor accessory protein, IL-33 is able to induce strong expression of Th2-like cytokines, thus balancing the Th1

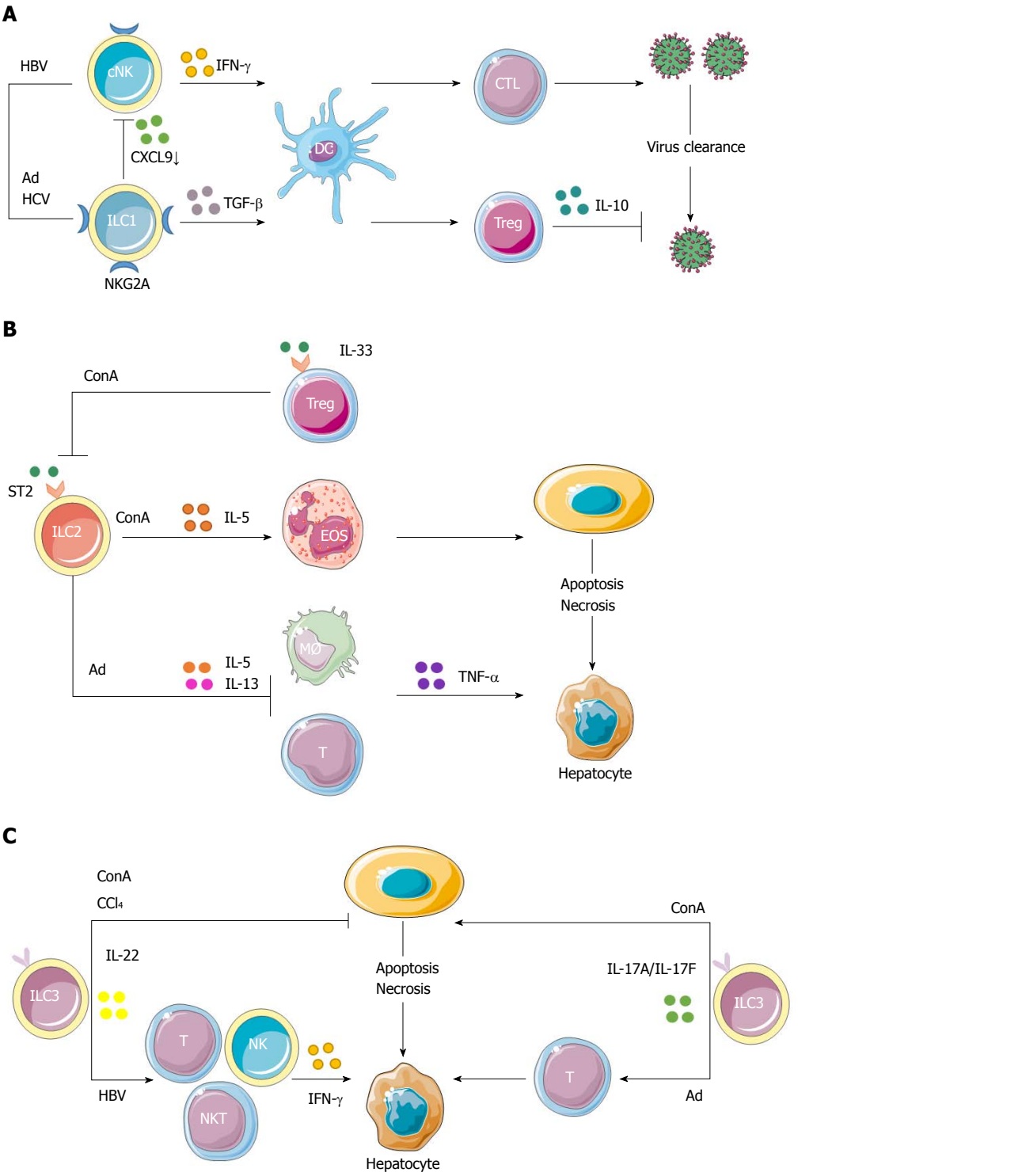

Figure 2 Protective and pathogenic roles of innate lymphoid cells in hepatic inflammation. A: cNK cells could produce IFN-γ to enhance the priming of $CD8^+$ T cells to clear HBV. The interactions of NK cells with hepatocytes *via* NKG2A inhibitory receptor could prime DCs to induce $CD4^+CD25^+$ Tregs, which would in turn up-regulate the expression of NKG2A on NK cells *via* IL-10 production, thus impairing the antiviral ability of NK cells. Because of increased expression of NKG2A on ILC1s in hepatic Ad as well as hepatitis C virus infection, ILC1s play a role in maintaining the liver as a tolerogenic site by inhibiting CXCL9 expression, which is required for the accumulation of cNK cells. This would further impair the activation of liver $CD103^+$ DCs, thus interrupting the proliferation of virus-specific $CD8^+$ T cells and the clearance of virus; B: In ConA-induced immune hepatitis, hepatic ILC2s could amplify inflammation through the expression of IL-5 to recruit eosinophils in response to IL-33 released upon liver tissue damage. The inflammatory activity of endogenous ILC2s in immune-mediated hepatitis might be regulated by IL-33-elicited $ST2^+$ Tregs. Besides, in Ad-induced viral hepatitis, a strong expression of ILC2s was induced by IL-33 to exert a protective role through down-regulation of the hepatotoxic cytokine TNF-α in T cells and macrophages. Both the proinflammatory and protective roles of ILC2s in hepatitis are part of IL-33 action; C: In immune hepatitis, ILC3-derived IL-22 has a protective role in ConA- and carbon tetrachloride-induced hepatitis, while IL-17 plays a pathological role in ConA-induced hepatitis. Besides, Notch-mediated IL-22 is an important mediator of the inflammatory response in HBV infection, being responsible for the recruitment of antigen-nonspecific inflammatory cells into the liver and subsequent liver injury. In Ad-induced acute hepatitis, the IL-17A/F signaling is critical for adaptive T response and is responsible for affected lymphocyte infiltration and hepatic inflammation. Ad: Adenovirus; cNK: Conventional natural killer; ConA: Concanavalin A; DC: Dendritic cell; HBV: Hepatitis B virus; IL: Interleukin; ILC: Innate lymphoid cell; NK: Natural killer; Tregs: T regulatory cells.

immune response[41,42]. Epithelial cells, hepatocytes and HSCs have been reported as the main sources of IL-33 in the liver[43,44]. The expression of IL-33 shows significant connection with chronic liver diseases, such as persistent viral infection[45-47], liver fibrosis[43] and liver failure[48].

It has been demonstrated that through interaction with ST2, IL-33 induces production of the inflammatory cytokine IFN-γ, as well as Fas-FasL interaction between hepatocytes and NKT cells in concanavalin A (ConA)-induced hepatitis, which is a well-established murine model of T-cell mediated hepatitis, resembling the pathology of immune-mediated hepatitis in humans[49,50]. Neumann *et al*[24] further demonstrated that IL-33-elicited ILC2s were also involved in the pathogenic process of murine immune-mediated hepatitis. In response to the release of IL-33 upon liver tissue damage induced by $CD4^+$ T cells, hepatic ILC2s were found to be poised to produce type 2 cytokines, including IL-13 and IL-5. Recruitment of eosinophils induced by IL-5 could be one mechanism by which ILC2 amplifies inflammation during immune hepatitis, and IL-13 was indicated to have a prominent role in chronic diseases[51]. Exogenous IL-33-elictied hepatic ILC2s appear to aggravate immune-mediated hepatitis, while the inflammatory activities of endogenous ILC2s might be regulated by IL-33-elicited $ST2^+$ Tregs, which showed strong expansion in immune-mediated hepatitis as well. These findings are consistent with those of the previous study that revealed IL-33/ST2 axis to exert a protective role in ConA-induced hepatitis by preventing Th1 and Th17 cell-mediated hepatic immune responses, promoting IL-4 production of $CD4^+$ liver-infiltrating T cells, elevating the total number of $CD4^+Foxp3^+$ Tregs together with affecting the expression of apoptotic or antiapoptotic proteins[52]. These results suggest that the proinflammatory role of ILC2s in immune-mediated hepatitis is part of the action mechanism of IL-33. Multiple modules of the immune response should be taken into consideration when investigating its overall protective or pathogenic effect on the liver.

What's more, the IL33/ST2 axis has also been shown to play a crucial role in driving antiviral $CD8^+$ and $CD4^+$ T cell responses[53,54]. On one hand, Liang *et al*[55] demonstrated that, as a newly discovered damage-associated molecular pattern molecule, IL-33 can promote innate IFN-γ production by γδT cells and NK cells. It could also modulate DC responses to enhance the plurifunctionality of antiviral T cells in lymphocytic choriomeningitis virus-induced hepatitis in mice, while it was also further demonstrated that ILC2s were not involved in this process[56]. On the other hand, IL-33 was able to directly engage multiple arms of immune mechanisms to mediate potent hepatoprotective effects in adenovirus-induced hepatitis, wherein strong CTL, $CD4^+$ Th and B lymphocyte responses share common characteristics with a number of hepatotropic viruses, including hepatitis A virus, HBV, cytomegalovirus, herpes simplex and Epstein-Barr virus. It significantly inhibited the expression of TNF-α in T cells and macrophages and induced a strong expression of IL-5- or IL-13-expressing-Lin^- nuocytes to further down-modulate the hepatotoxic cytokine TNF-α[57]. An increased number of Lin^-13^+ or Lin^-5^+ cells were found in the livers of Lin^- cells adoptively-transferred mice. Though the serum level of alanine aminotransferase and hepatic TNF-α presented a downward trend, there was no statistical significance compared with control groups[58]. These results also suggest that the potential protective role of ILC2s in viral hepatitis might only be one facet of the complex mechanisms of IL-33, but this still remains to be further elucidated (Figure 2B).

Group 3 ILCs

Dependent on RORγt and IL-7, ILC3s induce the production of IL-17 and IL-22 upon stimulation by IL-23 and IL-1β. IL-22 is a member of the IL-10 cytokine family and has a crucial role in inflammation, immune surveillance and tissue homeostasis. In the inflammatory context, IL-22 has both proinflammatory and protective properties[59,60]. The proinflammatory nature of IL-22 has been shown in mouse models with diseases such as psoriasis and rheumatoid arthritis[61,62]. In contrast, the protective role has been shown in inflammatory bowel disease[63], hepatitis[64] and pathogenic bacterial infection[65,66]. Hepatocytes are important targets of IL-22, for it can induce the expression of acute-phase proteins, several antiapoptotic proteins and mitogenic proteins, to protect cells against liver tissue damage[67-69]. IL-22 can also act on liver stem or progenitor cells, which are important in chronic and severe liver injury[70].

In ConA-induced acute immune hepatitis, the expression of IL-23 combined with activated Notch signaling resulted in an aryl hydrocarbon receptor (AHR)-dependent production of IL-22, as well as in an RORγt-dependent production of IL-17. IL-22 was shown to play a protective role, while IL-17 was shown to be critical for the pathogenesis in liver tissue[71]. The protective role of IL-22 in hepatitis was consistent with findings of a previous study that identified NKT and T cells, rather than ILC3s, as the main sources of IL-22[64]. Later, it was confirmed by Abe *et al*[72] that, combined with the suppression of IFN-γ from NKT cells induced by AHR, IL-22-producing ILC3s were also involved in the protective process in ConA-induced acute hepatic injury, as high IL-22 mRNA levels were found in $CD3^-Sca1^+Thy1^+$ cells rather than in $CD3^+$ T cells after stimulation by IL-23. Besides, the same results were obtained in *RORγt*$^{-/-}$ mice; specifically, there was almost no IL-22 production in the hepatic mononuclear cells of *Ahr*$^{-/-}$ or *Ahr* and *recombination activation gene* (*RAG*) double-negative mice, thus further suggesting that the major sources of IL-22 were both RORγt- and AHR-dependent ILC3s. In addition, the decreased frequency of IL-22-producing ILC3s (Lin^- $SCA\text{-}1^+$ $Thy1^{high}$ ILCs) was consistent with the severity of carbon tetrachloride-induced hepatitis of *RAG-2*$^{-/-}$**RORγt*$^{-/-}$ mice compared with that of *RAG-2*$^{-/-}$ mice[72].

Taken together, all the results of these studies considering RORγt$^+$ hepatic ILC3s in immune-mediated hepatitis suggest their protective roles against liver injury *via* IL-22 production. Compared with IL-22-producing Th17 cells, which can play a protective role against liver injury as well, hepatic ILC3s may be able to act in the early innate immune response stage[73]. On the contrary, IL-17, another ILC3s-derived cytokine, has shown a pathological role in ConA-induced hepatitis. The overexpression of IL-17A resulted in massive hepatocyte necrosis, and antiIL-17A blockage significantly ameliorated liver injury[74]. In addition, Lafdil *et al*[75] showed that liver injury was alleviated in ConA-induced hepatitis among IL-17-deficient mice.

On the other hand, however, IL-22 was identified as a potent mediator of the inflammatory response in HBV infection, following the recognition of HBV by T cells in the liver[67,76]. It was further confirmed that inhibition of Notch signaling *in vivo* would lead to decreased ILC22 and LTi4 cells, along with down-regulated expressions of IL-22 and related proinflammatory cytokines and chemokines in the liver. As a result, subsequent liver injury was alleviated due to blockage of the recruitment of antigen-nonspecific inflammatory cells into the liver, without affecting HBV antigen production in HBV infection[77]. These results suggest the potential proinflammatory role of Notch-mediated IL-22 and provide a new potential therapeutic approach for the treatment of HBV. What's more, intrahepatic early IL-17 was found to be important for activating antigen presenting cells in viral infection, but the sources and regulation of IL-17 surges were not well defined at first[78]. It was further shown that ILC3s, including a large proportion of NKP46$^-$ ILC3s and a small part of the CD4$^+$ LTi cells, secreted IL-17A and IL-17F shortly after adenovirus infection, in addition to γδT cells. In adenovirus-induced acute hepatitis, the IL-17A/F signaling was found to be critical for adaptive T response and was responsible for affected lymphocyte infiltration and hepatic inflammation, except in viral clearance. The study also revealed the existence of the compensatory IL-17F production for IL-17A deficiency underlying the previous contradictory result that IL-17A deficiency did not appear to thwart T cell activation and liver inflammation[64,79]. Though there have been studies showing Th17-derived IL-17 causes liver damage by IL-23 activation, the role of ILC3-derived IL-17 remains to be further clarified in chronic infection models, such as for lymphocytic choriomeningitis virus and HBV infections[80]. To conclude, the downstream effector cytokines of ILC3s may exert both proinflammatory and protective roles according to the specific microenvironment, and more studies are required to clarify their explicit role in liver inflammation (Figure 2C).

LIVER FIBROSIS

Group 1 ILCs

NK cells can directly decrease the proliferation and activation as well as induce cell cycle arrest of HSCs through IFN-γ[81,82]. They can also induce apoptosis of activated HSCs through the TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand pathways[83,84]. These interactions between NK cells and HSCs are regulated by NK cell receptors and cytokines. The activation of HSCs in response to hepatocyte damage leads to increased NK cell stimulation and decreased NK cell inhibition. Firstly, increased amounts of retinoic acid derived from activated HSCs was found to be associated with elevated expression of RAE-1, a ligand for the NKG2D-activating receptor. Together with MICA, RAE-1 could promote the killing of activated HSCs by NK cells[85,86]. The NKp46 and NKp30 activating receptors have also been shown to be involved in the amelioration of liver fibrosis by inducing HSC killing by NK cells, in both humans and mice[87,88]. Secondly, engagement of Ly49 inhibitory NK cell receptors was found to be reduced by the mechanism of siRNA-mediated silencing, as a result of down-regulated major histocompatibility complex (MHC) class I in activated HSCs[89,90]. Besides, elevated surface expression of TRAIL in NK cells *via* IFN-α, simultaneous with increased expression of TRAIL receptors, in activated HSCs could also enhance HSC killing by NK cells[91,92]. Instead, tumor growth factor (TGF)-β down-regulates the surface expression of NKG2D and 2B4 to suppress the antifibrotic role of NK cells[93]. Whether trNK cells exert a protective role in liver fibrosis remains unclear. As a member of the IFN-γ-producing group 1 ILCs, these cells may contribute to the activation of NK cells by their production of IFN-γ, which is crucial for the antifibrotic roles of NK cells[81].

Group 2 ILCs

In the study by Marvie *et al*[43], the over-expression of IL-33 was shown to be closely associated with hepatic fibrosis, in both human and mouse cases. Besides, type 2 cytokines including IL-4 and IL-13 have been considered as representatives of the most potent fibrogenic factors[94]. As the major sources of Th2-type cytokines, ILC2s, which also require IL-33 for activation, were proposed as potent profibrogenic factors in hepatic fibrosis[95]. It has been demonstrated that, in response to ST2-dependent signaling owing to chronic hepatocellular stress and tissue damage, IL-33 release leads to accumulation and activation of IL-13-producing liver resident ILC2s. The downstream effector cytokine IL-13 can further trigger the activation and transdifferentiation of HSCs in an IL-4Ra- and STAT6-dependent manner to induce potent fibrogenic responses, suggesting a pathogenic capacity of ILC2s in the context of the tissue damage response[22]. In parallel, the study of human liver fibrosis by Forkel *et al*[96] has identified primary hepatocytes, HSCs and Kupffer cells as cellular sources of IL-33 and TSLP, which could further potentially cause the accumulation of ILC2s in fibrotic livers following TLR3-activation, as a model for hepatitis C infection. There was also a direct correlation found between the increase in

frequency of intrahepatic ILC2s and the severity of liver fibrosis. The induction of IL-13 by intrahepatic ILC2s in response to IL-33 and TSLP was also confirmed, suggesting the possibility of a similar mechanism in humans and mice[96]. These results provide an avenue for investigation into the application of serum IL-33 as a possible noninvasive diagnostic biomarker for uncovering early inflammatory and fibrogenic events. Furthermore, targeting ILC2s may represent a novel therapeutic strategy for liver fibrosis treatment.

Group 3 ILCs

As one of the ILC3s' downstream effector cytokines, IL-22 has been shown to promote the survival and proliferation of epithelial cells (*e.g.*, hepatocytes), suggesting its possibility of involvement in liver fibrosis[64,68]. Upon binding to IL-22R1 and IL-10R2 on HSCs, IL-22 can induce senescence of the HSCs following the activation of a STAT3/SOCS3/p53 signaling axis, which represents an important strategy to ameliorate liver fibrosis[97]. It could also inhibit HSC apoptosis, without affecting HSC proliferation. By means of enhancement of *in vivo* clearance of senescent HSCs, most probably by NK cells, simultaneous to reduction of released tissue inhibitor of metalloprotease 2 to promote MMP activities and down-regulate the deposition of collagen, the senescence of activated HSCs played an important role in limiting liver fibrosis[86,98]. The expression of α-smooth muscle actin was also down-regulated in response to IL-22, but this effect was not associated with senescent HSCs[97]. Besides, elevated systemic IL-22 level - independent of age, liver-related complications, C-reactive protein, creatinine and model for end-stage liver disease score - could be predictive for reduced survival prognosis in patients with liver cirrhosis. Thus, it is possible that systemic IL-22 level could be applied as a negative indicator for evaluating the prognosis of advanced liver cirrhosis[99]. Though no direct evidence has linked ILC3s with liver fibrosis, ILC3s may exert a protective role since they are the source of IL-22. Nonetheless, considering IL-22 can be produced by Th17 cells as well, it is important to use specific gene knock-out mice to determine which type of cell plays the pivotal role.

In experimental liver fibrosis, upon stimulation of IL-17A, both HSCs and Kupffer cells could produce TGF-β, TNF-α and IL-6 following the activation of STAT3 and nuclear factor-κB. Accordingly, mouse models with IL-17A and IL-17RA deficiency have displayed reduced liver fibrosis, suggesting a profibrotic role[100]. In addition, IL-17A can also exert an antifibrotic effect in normal fibroblast cultures directly, by down-regulating the expressions of collagen and connective tissue growth factor, which was shown to be impaired in the isolated primary fibroblasts from patients with scleroderma[101]. Considering the complicated and versatile effects in fibrosis, it might be necessary to determine the exact roles of such cytokines in different stages of liver fibrosis (Figure 3).

LIVER CANCER

Group 1 ILCs

Considering the potent tumor surveillance properties of NK cells, a group of NK cell-associated genes in hepatocellular carcinoma (HCC) tissues was positively associated with prolonged survival[102]. Evidence of dysfunction of NK cells in HCC has been observed, as well, suggesting a strong connection between NK cells and HCC progression[103].

Although there currently is no direct evidence revealing connections between ILC1s and liver tumor immunity, the effects of their secreted cytokines have been extensively investigated, among which IFN-γ was shown to have prominent antiproliferative, antiangiogenic and proapoptotic effects against cancer cells[104-106]. This cytokine can promote the up-regulation of MHC molecules to induce the priming as well as antigen processing and presentation of professional antigen presenting cells, and has been shown to increase the immunogenicity of tumor cells, thereby enhancing antitumor responses[107,108]. In addition to promoting the polarization of $CD4^+$ T cells into Th1 cells, it can also boost the responses of macrophages, NK cells and CTLs against tumor tissues[109,110]. TNF-α, another cytokine secreted by ILC1s, can also play an antitumor role by interfering with angiogenesis, cellular growth and migration. Further, it can induce the recruitment of macrophages and DCs, as well as the generation of CTLs, leading to a strong antitumor immune response[111,112]. Combined with the previously reported research findings, a recent study which showed the $NK1.1^+CD49a^+CD103^+$ ILC1-like cells could lyse tumor cells, dependent on the activation of granzyme B and TRAIL, in an oncogene-induced cancer model also supports the protective function of ILC1s in antitumor immunity[113]. However, its protective function can be hampered in cancer patients, as ILC1s of acute myeloid leukemia patients were found to be dramatically impaired in their production of IFN-γ and TNF-α compared to those of healthy control subjects[114].

It was also demonstrated that both IFN-γ and TNF-α could play ambiguous roles in cancer immunity. The protumor function of IFN-γ involves increased proliferative and antiapoptotic signals, as well as escape of the tumor cells from recognition and cytolysis by CTLs and NK cells[115]. TNF-α is also involved in tumor formation, growth and spread considering its versatile impacts on the expression of angiogenic and growth factors, cytokines, adhesion receptors and proteases[111,116,117]. Recently, Gao *et al*[118] demonstrated that $CD49a^-CD49b^+Eomes^+$ NK cells could convert into intermediate $CD49a^+CD49b^+Eomes^+$ type 1 innate lymphoid cells (intILC1s) and $CD49a^+CD49b^-Eomes^-$ ILC1s in tumor microenvironment in a TGF-β signaling-dependent manner. Strikingly, distinguishable from the potent tumor surveillance properties of NK cells, intILC1s and ILC1s were incapable of controlling local tumor growth

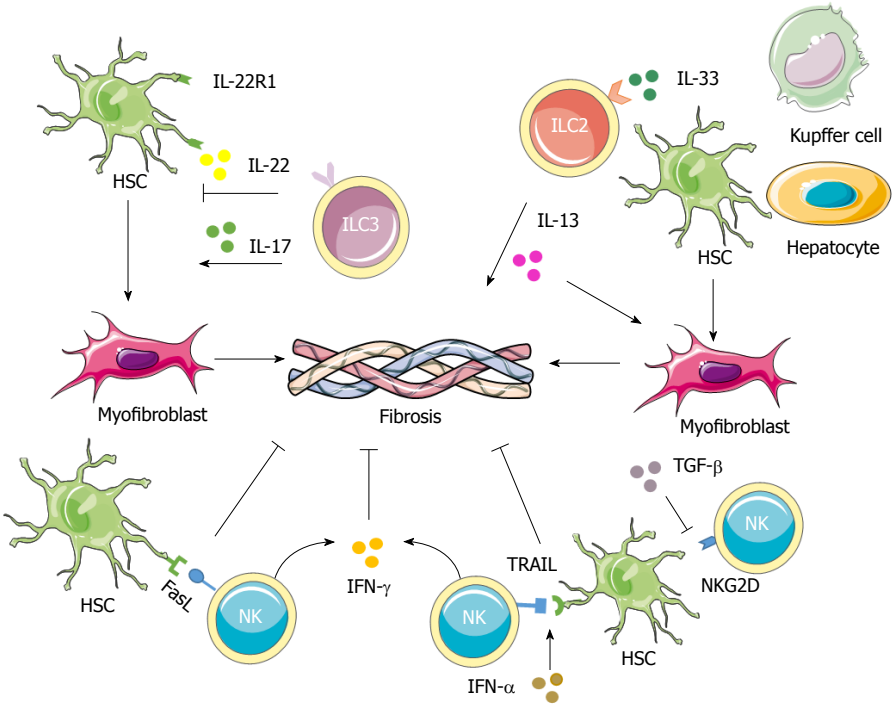

Figure 3 Contributions of innate lymphoid cells in liver fibrosis. NK cells can decrease the proliferation and activation as well as induce cell cycle arrest of HSCs through IFN-γ. They can also induce the apoptosis of activated HSCs through the TRAIL and Fas ligand pathways. The expression of RAE-1, which is the ligand for the NKG2D activating receptor, is increased on activated HSCs, thus promoting killing by NK cells. IFN-α could increase the surface expression of TRAIL on NK cells to enhance HSCs killing by NK cells, while TGF-β down-regulates the surface expression of NKG2D and 2B4 to suppress the antifibrotic role of NK cells. In both human and mouse cases, IL-33 released from hepatocytes, HSCs and Kupffer cells in response to chronic hepatocellular stress leads to the accumulation and activation of IL-13-producing liver resident ILC2s *via* ST2-dependent signaling. IL-13 further triggers the activation and transdifferentiation of HSCs into myofibroblasts, to induce potent fibrogenic responses. ILC3s play more complicated roles in fibrosis. On one hand, ILC3s exert an antifibrotic effect by inducing fibroblasts senescence through IL-22 signaling and by down-regulating the expressions of collagen and CTGF through IL-17 signaling. On the other hand, IL-17 can promote inflammation and induce activation of fibroblasts, indicating a profibrotic role for the ILC3s. CTGF: Connective tissue growth factor; HSC: Hepatic stellate cell; IL: Interleukin; IFN: Interferon; NK: Natural killer; TGF: Tumor growth factor; TRAIL: TNF-related apoptosis-inducing ligand.

and metastasis, uncovering an unknown mechanism by which tumors can escape surveillance by the innate immune system. This study also provided a new insight into the phenotypic and functional plasticity of tumor group 1 ILCs, while the precious roles and interactions of ILC1s in tumor microenvironment - especially in the liver - still needs to be further elucidated[118,119].

Group 2 ILCs

Considering the potential profibrogenic properties of ILC2s elicited by IL-33 in hepatic fibrosis under the circumstances of tissue damage, it is possible that these cells are involved in the progression from liver fibrosis to cancer as well. The precise role of ILC2s in carcinogenesis remains unclear; however, it can be supported by the evidence that has emerged from studies addressing factors that trigger their activation and proliferation, as well as their downstream effector molecules.

When it comes to the liver, IL-33 was shown to be involved in the initiation of cancer, based on a previous study wherein increased expressions of Th2 cytokines and hepatic IL1RL1 mRNA encoding ST2 were detected in a subgroup of patients at the time of diagnosis of biliary atresia[120]. ILC2s were further identified as important mediators of the IL-33-depedent proliferative response for their production of high levels of IL-13, which in turn promoted cholangiocyte proliferation and epithelial hyperplasia in mice by involving the activation of IL-4R and the downstream target Stat6. Administration of IL-33 with constitutive activation of AKT and Yes-associated protein in biliary epithelium would lead to the development of cholangiocarcinoma, which resembles both the morphological and biochemical features of human disease in a mouse model of experimental carcinogenesis[121,122]. Thus, the activation of the IL-33/ILC2s/IL-13 circuit may promote epithelial repair, and the disruption of IL-33 or other elements of the paracrine circuit may constitute potential new therapeutic targets against cholangiocarcinoma. Furthermore, the level of IL-33 was found to be increased in patients with HCC as well[123].

IL-33 can also increase the intratumor accumulation of myeloid-derived suppressor cells (MDSCs), which require arginase and nitric oxide synthase II from IL-13 for their activation[124,125]. Together with the pro-angiogenesis process, MDSCs can also produce TGF-β to support tumor progression[126]. Besides, IL-13 can induce the polarization of macrophages to the M2 phenotype, and the production of growth and angiogenic factors to promote tumor initiation, progression and metastasis[127,128]. It has also been demonstrated that

amphiregulin, another cytokine secreted by ILC2s, could enhance the activities of Tregs *in vivo*, which would further inhibit antitumor immune responses induced by DC vaccination[129,130].

IL-33 also plays a role in antitumor immune responses *via* effector functions of both $CD8^+$ T cells and ILC2s, dependent on its dose[131,132]. The latter was confirmed in a study by Kim *et al*[133], which demonstrated that ILC2s were involved in IL-33-mediated antitumor responses. A massive amount of CXCR2 ligands released from ILC2s interacted with CXCR2 expressed by tumor cells by means of a dysfunctional angiogenesis/hypoxia/reactive oxygen species axis triggered by IL-33, subsequently leading to the apoptosis of active tumor cells[133]. Taking these results into consideration, ILC2s could exert both immune suppression and antitumor functions according to different tumor microenvironments, while its precious role, especially in the context of human livers, remains to be further confirmed.

Group 3 ILCs

The protective role of ILC3s has been directly revealed for its contribution to the formation of protective tumor-associated tertiary lymphoid structures in non-small cell lung cancer (NSCLC)[134]. The ILC3s are able to up-regulate adhesion molecules in the tumor microenvironment to enhance leukocyte invasion, and have been characterized as important mediators of the efficacy of a combination therapy of chemotherapy and tumor antigen-targeted monoclonal antibodies[135]. Though there is no direct evidence linking ILC3s and liver cancer, the connection can be inferred according to their role in colorectal cancer, as well as the ambiguous roles of their downstream effector cytokines, including IL-22 and IL-17.

It has been demonstrated that ILC3-derived IL-22 is crucial for promoting bacterial inflammation-induced colorectal cancer in $Rag^{-/-}$ mice through the activation of epithelial cells *via* STAT3 signaling[136]. Furthermore, deficiency of soluble IL-22 binding protein (IL-22BP) secreted by immature DCs was found to be associated with increased colitis-associated colon cancer due to the aberrant proliferation induced by IL-22. However, IL-22 was demonstrated as important for colonic epithelial cell repair in the early stage of colitis, using the same model; in particular, $IL22^{-/-}$ mice were shown to have enhanced cancer development[137]. These results suggest that IL-22 is crucial for regulating intestinal tissue repair during the peak of damage, while prolonged IL-22 in the recovery phase would be expected to favor tumorigenesis.

Paralleling the dual effects of IL-22 on tumorigenesis of colitis-associated colon cancer, IL-22 has also been reported to induce tissue regeneration or tumorigenesis and metastasis in the liver. Firstly, characterized with the protective role of hepatocyte proliferation and tissue regeneration during hepatitis and after hepatectomy, the functions of IL-22 may be exploited in liver cancer, as suggested by the significant up-regulation of IL-22 detected in human HCC tumor-infiltrated leukocytes[70,138,139]. Besides, there is a positive correction between IL-22 expression and the oncogenesis and staging of tumors, according to the finding that both IL-23 and IL-22BP are highly expressed in tumor tissue[68]. Secondly, though the induction of MMP enables IL-22 to protect against tumor formation in chronic liver fibrotic diseases, by the same mechanism it can increase the metastatic capacity of established tumor cells by digesting ECM, invading surrounding tissue and escaping the primary site. This has been shown in the A549 lung carcinoma cell line and pancreatic cancer, while whether the same mechanism also exists in hepatic tumor tissue remains unknown[97,140,141].

The same balance also exists in the antiviral activity and associated oncogenesis. IL-22 disturbs the establishment of chronic inflammation to prevent liver cancer. As was shown in acute infection of HBV, IL-22 acts as the mediator of an acute phase reaction to clear the virus *via* the recruitment of T cells[142]. However, it plays a contrary role in the progressive diseases, as IL-22 level was elevated and high serum IL-22 level indicated a poor prognosis both in patients with HBV and hepatitis C virus-associated HCC, suggesting that expression of IL-22 during progressive disease may reflect increased aggressiveness of HCC instead of predisposal to cirrhosis[76,143].

IL-22 can also influence the outcome of tumorigenesis by the mechanisms of pro- and antiinflammatory functions, angiogenesis, epithelial-mesenchymal transition, dysplasia and metabolic functions that remain less clear in the liver[142]. All these results suggest whether the effect of IL-22 is tumorigenic or antitumorigenic seems to depend on the stage of their responses and the specific tumor microenvironment.

$Thy1^+IL\text{-}23R^+$ ILC3s are important for IL-23-induced initiation of gut tumorigenesis, as substantial inhibition of tumorigenesis in $RAG^{-/-}*IL17^{-/-}$ double knock-out mice provided evidence for an important contribution of IL-17 expression in ILC3s, which consistently occurred before the recruitment of overt inflammatory infiltrates[144]. When it comes to liver, the connection between IL-17 and angiogenesis has emerged in the context of HCC[145]. Besides, it has been shown to have protumor activity in proliferation, immune-resistance, tumorigenesis and metastasis as well[146]. On the contrary, IL-17 also plays a vital role in antitumor activity *via* the stimulation of tumor-specific CTLs, which were associated with establishment of a tumor-protective immunity in hematopoietic cancer[147]. These results also lead to the suggestion that there is a balance between protective CTLs' formation during the acute phase of hepatitis and angiogenic activity during the chronic phase, which would determine the outcome of tumors.

Overall, ample evidence has pointed towards ILC3s having an important role in tumor progression. The

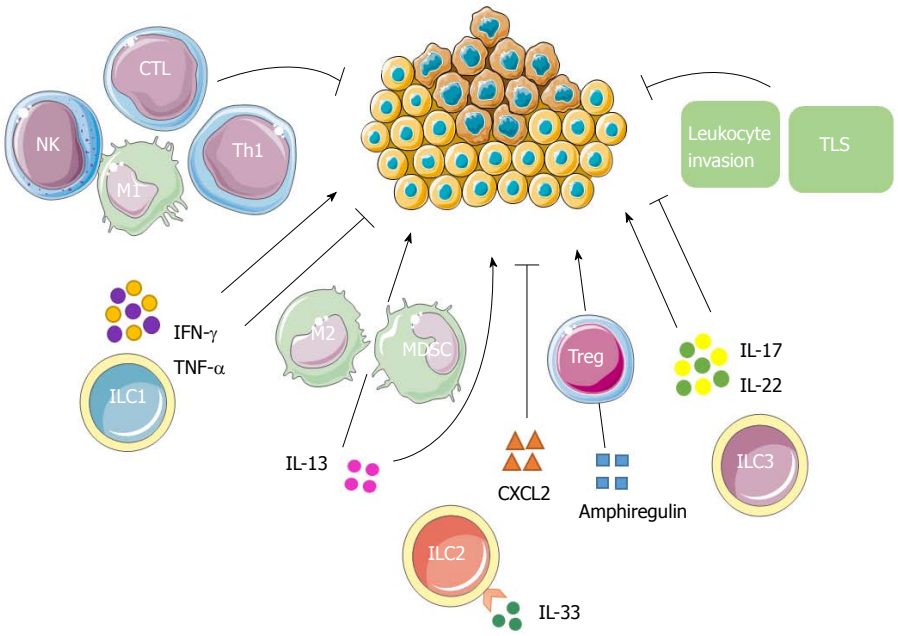

Figure 4 Possible innate lymphoid cell interactions in liver tumor immunity. ILC1s have both antitumor and protumor effects according to the properties of cytokines secreted. IFN-γ and TNF-α have antiproliferative, antiangiogenic and proapoptotic effects against cancer cells. In addition to promoting the polarization of $CD4^+$ T cells into Th1 cells, they can also boost the responses of macrophages, NK cells and CTLs, leading to a strong antitumor response. On the contrary, their ambiguous roles enable them to enhance tumor formation, growth and spread. ILC2s may contribute to tumor progression either directly through the tumorigenic effects of IL-13, or indirectly by stimulating M2 macrophages and MDSCs through IL-13. The production of amphiregulin suggests that ILC2s may further inhibit antitumor immune responses either directly or via stimulation of Tregs. IL-33 could also induce the secretion of a massive amount of CXCR2 ligands from ILC2s as well as create a tumor microenvironment wherein tumor cells express CXCR2, leading to apoptosis of active tumor cells. ILC3s might promote antitumor responses by enhancing leukocyte invasion, promoting TLSs' induction and through the antitumor effects of IL-17 and IL-22. Conversely, ILC3s may promote tumor formation and progression by IL-17 and IL-22 as well according to the phase of responses and specific tumor microenvironment. CTL: Cytotoxic T lymphocyte; IL: Interleukin; ILC: Innate lymphoid cell; IFN: Interferon; MDSC: Myeloid-derived suppressor cell; NK: Natural killer; Th: T helper; TLS: Tertiary lymphoid structure; TNF: Tumor necrosis factor; Tregs: T regulatory cells.

elements consisting of specific tumor microenvironment as well as the timing of responses count when considering their ambiguous roles, while their explicit functions in humans, especially in human liver cancer, are incompletely understood and remain to be fully elucidated (Figure 4).

CONCLUSION AND FUTURE PERSPECTIVES

The recent research studies on the roles of ILCs in development of chronic liver diseases have made great progress, especially for hepatitis and liver fibrosis. Different or even the same ILC subsets have shown complex functions at a certain stage of chronic liver diseases. Also, the same ILC subsets have exhibited both pathological and protective functions during the dynamic development of chronic liver diseases. When considering their effects on liver, both the downstream effector cytokines and the molecules involved in the upstream signaling must be taken into consideration simultaneously, and more research is required to further elucidate the underlying mechanisms and signaling pathways. Apparently, there is a balance between the protective and pathological properties of ILCs, according to the specific liver tissue microenvironment at different stages of liver diseases, whereby effector cytokines, surrounding interaction cells and functional state of cell receptors vary remarkably. What's more, the basis of the functions of ILCs and their downstream effector cytokines in hepatitis and liver fibrosis can represent the foundation of future research interests for investigating their roles in tumorigenesis.

Different methods have been applied to detect ILCs and measure their activities. Intrahepatic as well as peripheral blood mononuclear cells are isolated for further *in vitro* staining with fluorescence-labeled antibodies according to the cluster of differentiation on the surface of different ILC subsets and intracellular contents. Flow cytometry is further applied to detect the frequency and cellularity of ILCs and analyse the expression of their transcription factors and effector cytokines induced by PMA/ionomycin once they have been sorted *in vitro*. By observation of the differences of these factors between patients with chronic liver diseases and healthy control groups, their changes before and after the inducing factors and their consistency with liver injury, the researchers could validate the activities and functions of ILCs in the liver[23,38]. Considering limited accessibility of primary intrahepatic ILCs, the expansion of cell lines of primary intrahepatic ILCs is also an alternative to assess the function of this

small cell population and to seek their secretion profile through the stimulation of PMA/ionomycin[96]. Besides, there have been studies exploring the roles of ILCs in initiation of liver injury including both hepatitis and liver fibrosis by the mechanism of *in vivo* depletion of ILCs using specific antibodies[22,73] or targeted transcription factor gene-deficient mice[72]. The protective or pathological roles of ILCs are determined by comparison of the severity of liver injury before and after the depletion of ILCs as indicated by histological analysis of liver tissue and expression of liver injury serum biomarkers as well as inflammatory cytokines in *RAG1*$^{-/-}$ mice which are reconstituted with CD4^{+} T cells. *In vivo* experiments to distribute the signaling pathway of ILCs through the blockade of upstream cytokines and surface receptors of ILCs *via* targeted gene-knock mice are also important methods, in which the expansion of ILCs and their expression of transcription factors and downstream effector cytokines are further detected by flow cytometry and quantitative real-time PCR analysis[24,52]. Additionally, the activities of ILCs could also be monitored by transfer experiments, in which purified ILCs sorted by MACS/FACS are adoptively transferred into recipient mice before the challenge of stimulus including ConA and carbon tetrachloride to further investigate the function of ILCs in the liver[22,24,58].

There still remains a lot to be fully elucidated. Firstly, the functions of some ILC subsets at a particular stage of chronic liver diseases have only been inferred by their downstream effector cytokines, while lacking direct and potent evidence. Secondly, given their distribution characteristics, evidence with regard to the functions of ILCs in tumorigenesis is emerging from studies that have mostly investigated chronic inflammation and the procarcinogenic role of secreted cytokines in skin, gut and lung, and less so for the liver. Thirdly, as the innate counterpart of CD4^{+} Th cells, the same effector cytokines can be produced by both adaptive lymphoid cells and ILCs. It is important to identify the sources of effector cytokines, while the results from the most recent *in vivo* studies were obtained from *RAG*$^{-/-}$ mice or antibodies that are specific to ILCs' genes leading to broad immune deficiencies. Thus, it is necessary to apply ILCs' specific gene-knockout or transgenic models to reveal the precise and direct actions of each in the liver.

New strategies targeting ILCs have been designed for diagnosis and treatment, to prevent or stop the progression of chronic liver diseases. The inhibition of NKG2A receptors on ILC1s to further promote robust CD8^{+} T cell responses has been considered a potential therapeutic strategy against persistent liver pathogens in patients with hepatitis. Besides, for liver fibrosis treatment, serum IL-33 may be a possible noninvasive diagnostic biomarker for uncovering early inflammatory and fibrogenic events. Targeting ILC2s may represent a novel strategy as well. Further in-depth studies to elucidate the distinct and explicit effects of each of the ILC subsets at different stages of chronic liver diseases are required in order to promote the exploration and realization of their therapeutic potency.

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MINIREVIEWS

Laparoscopic gastrojejunostomy for gastric outlet obstruction in patients with unresectable hepatopancreatobiliary cancers: A personal series and systematic review of the literature

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Abstract

The major symptoms of advanced hepatopancreatic-biliary cancer are biliary obstruction, pain and gastric outlet obstruction (GOO). For obstructive jaundice, surgical treatment should de consider in recurrent stent complications. The role of surgery for pain relief is marginal nowadays. On the last, there is no consensus for treatment of malignant GOO. Endoscopic duodenal stents are associated with shorter length of stay and faster relief to oral intake with more recurrent symptoms. Surgical gastrojejunostomy shows better long-term results and lower re-intervention rates, but there are limited data about laparoscopic approach. We performed a systematic review of the literature, according PRISMA guidelines, to search for articles on laparoscopic gastrojejunostomy for malignant GOO treatment. We also report our personal series, from 2009 to 2017. A review of the literature suggests that there is no standardized surgical technique either standardized outcomes to report. Most of the studies are case series, so level of evidence is low. Decision-making must consider medical condition, nutritional status, quality of life and life expectancy. Evaluation of

the patient and multidisciplinary expertise are required to select appropriate approach. Given the limited studies and the difficulty to perform prospective controlled trials, no study can answer all the complexities of malignant GOO and more outcome data is needed.

Key words: Duodenal obstruction; Gastrojejunostomy; Gastroenterosmy; Gastric outlet obstruction; Gastric bypass; Laparoscopy; Laparoscopic surgery; Sytematic review

Core tip: Both non-operative endoscopic approach and surgical treatment are available for palliative treatment of gastric outlet obstruction due to advanced hepatopancreatic-biliary cancer. Stent is usually preferred in patients with poor general condition or short life expectancy. Laparoscopic gastrojejunostomy is a feasible, safe and efficient technical option. Given the limited studies, we performed a systematic review of laparoscopic gastrojejunostomy in patients with advanced hepatopancreatic-biliary malignancy. Clinical prospective trials comparing different approaches with adequate sample size are warranted.

Manuel-Vázquez A, Latorre-Fragua R, Ramiro-Pérez C, López-Marcano A, De la Plaza-Llamas R, Ramia JM. Laparoscopic gastrojejunostomy for gastric outlet obstruction in patients with unresectable hepatopancreatobiliary cancers: A personal series and systematic review of the literature. *World J Gastroenterol* 2018; 24(18): 1978-1988 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1978.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1978

INTRODUCTION

Obstructive jaundice, gastric outlet obstruction (GOO) and tumor-associated pain are the major symptoms of advanced hepatobiliary-pancreatic (HPB) cancer. Usually these cancers are not resected because of infiltration of local structures or disseminated disease. Today, these complications can be managed with endoscopic stenting and percutaneous treatment, which have lower rates of associated morbidity; thus, surgical strategies have decreased.

Around 70% of cases of advanced HPB cancer present obstructive jaundice[1,2], which is the most common symptom[3-5] To resolve jaundice in unresectable or metastatic patients, endoscopic or percutaneous biliary stent is accepted as the gold standard[6]. Surgical treatment of biliary obstruction should be considered in persistent stent-problems, such as recurrent cholangitis or recurrent obstructive jaundice[6]; however laparoscopic surgery for biliary bypass is not a standard procedure[1,7]. Furthermore, the role of surgical pain relief in these patients seems to be marginal nowadays[6].

Finally, there is no consensus about the role of surgery in the management of malignant GOO. This clinical syndrome is characterized by abdominal pain, weight loss, nauseas and vomiting, due to the mechanical obstruction, and may be caused by gastric, duodenal, HPB or extraluminal diseases; therefore, the treatment depends on underlying cause[8]. In recent decades, 50%-80% of cases have been attributed to malignancy. GOO may develop in up to 20% of patients with advanced HPB disease[4,9-14]. The aim of GOO treatment is to reestablish oral intake by restoring gastrointestinal continuity.

Decision-making with regard to palliative treatment of malignant GOO due to advanced HPB cancer has become more complex in recent years. Traditionally, open gastrojejunostomy (OGJ) was the only option[11,15]. In the 1990s, endoscopic duodenal stents were introduced. In the last few years, laparoscopic gastrojejunostomy (LGJ) has emerged as a feasible and safe option that offers improved morbidity and mortality rates compared with the open surgical approach[3]. As can be seen, then, several options are available and there is no current gold standard[9].

The literature on GOO focus on gastric disease and mixes different causes with different prognoses. This means that, the level of evidence in patients with HPB malignant diseases is low and data on the laparoscopic approach to GJ for malignant GOO due to advanced HPB cancers are limited.

Our aim in this paper is to review various aspects of the management of malignant GOO due to advanced HPB cancer. Focusing on the laparoscopic approach for gastroenteroanastomosis, we perform a systematic review of the literature and a retrospective review of our personal series of laparoscopic GJ for the treatment of malignant GOO due to advanced HPB cancer.

ROLE OF PROPHYLACTIC GASTROJEJUNOSTOMY

A cancer may be found to be unresectable during preoperative staging examinations. Only some 20% of HPB neoplasms are found to be resectable[16-18]. Despite the indications of preoperative staging radiological and endoscopic images, between 8% and 33% of patients are found to be unresectable on laparotomy[19]. This means that surgeons may be encounter this situation intraoperatively and must decide whether to perform prophylactic GJ. This decision should be based on the probability of GOO; between 10%-15% of patients develop GOO at a later stage[3,11,20].

Gurusamy *et al*[2] report no differences in overall survival, postoperative morbidity and mortality, quality of life (QOL) or length of stay (LOS). This Cochrane review included two RCTs assessing the role of prophylactic GJ in unresectable periampullary cancer[21,22]. The authors reported a long-term GOO incidence of 27.8% in patients with advanced HPB cancer who did not undergo

prophylactic GJ and concluded that prophylactic GJ may not be necessary in all patients with advanced HPB malignancy undergoing laparotomy[2].

PALLIATIVE TREATMENT OF GOO

Physicians may also find a patient with uncontrolled vomiting and a diagnosis of advanced HPB malignancy. Palliative treatment should be offered to relieve the symptoms of GOO and ultimately to improve patient QOL. Palliative treatment is mandatory when the vomiting is uncontrolled.

Stent vs palliative surgery

Traditionally, OGJ was the only option for the treatment of malignant GOO[11,13,15]. Since 1992, several studies have described the use, safety and efficacy of self-expandable metallic stents (SEMS)[10,23-33]. Thus, several options are currently available and there is no established gold standard.

The literature on palliative GJ show good functional outcomes and symptoms relief in up to 70% of patients and reduced re-intervention rates, but it is associated with postoperative complications, such as delayed gastric emptying (DGE)[8,12,14,34,35]. For its part, palliative endoscopic treatment is a well-established procedure today and is considered a valid alternative for avoiding surgery. The endoscopic approach is associated with shorter length of stay (LOS), faster initial relief and shorter time to oral intake, but also with greater symptom recurrences and risk of stent migration[8,12-14,36-39].

The current literature mixes together different etiologies, and even includes benign causes such as superior mesenteric artery syndrome, peptic ulcer stenosis, chronic pancreatitis or annular pancreas, different grades of GOO, and prophylactic and palliative treatments[40-44]. Kohan *et al*[45] report the results of surgical palliative treatment for pancreatic cancer; but they mixed elective bypass for the treatment of symptomatic malignant GOO together with and prophylactic GJ in advanced HPB cancer patients undergoing surgery for biliary obstruction.

Table 1 shows the results of previous systematic reviews and meta-analysis comparing endoscopic duodenal stent *vs* GJ for the treatment of malignant GOO, including both gastric and advanced HPB cancers and other metastastic cancers. Minata *et al*[8], Nagaraja *et al*[13] and Ly *et al*[38] have demonstrated shorter LOS and faster oral intake with endoscopic palliative treatment, but lower re-intervention rates with OGJ. No differences in survival or major complications were found. Nagaraja *et al*[13] concluded that the endoscopic approach minimizes pain, hospitalization, and physiologic stress to the patient, which are the main goals of palliation.

Decisions regarding the best therapeutic strategy for individual patients with malignant GOO due to advanced HPB cancer should be based on the performance and medical condition, the extent of the cancer, the prognosis, their quality of life and expectancy, and the availability and likely success of each treatment option[36,46,47].

Depending on the medical condition, one of the main factors to consider is nutritional status; thus, hypoalbuminemia is considered as a risk factor for GJ whether the disease is benign or malignant[48]. Surgeons should correct this situation if surgical palliation is the aim and at least 1-2 wk of nutritional treatment should be considered in order to decrease the risk of postoperative complications[48]. According Sasaki *et al*[49], poor performance status should be considered as additional risk factor.

With regard to the extent of the cancer, the presence of carcinomatosis with ascites has been reported as an independent predictive factor for poor clinical success of stent placement, without any differences in stent patency[50].

The choice of palliative GJ or endoscopic enteral stent should consider the life expectancy of patients and the likelihood of recurrent GOO after stenting. As regards the prognosis of malignant disease, in the SUSTENT study, Jeurnink *et al*[12], concluded that palliative GJ is the treatment of choice in patients expected to live two months or longer, whereas stent is preferable for patients with a life expectancy below this figure. This conclusion is based on the finding that surgery was more effective than endoscopic stent after a follow-up of two months[12]. Recurrent obstruction due to tumor ingrowth into stent or stent migration has been reported in 17%-27% of patients with endoscopic stent[4,51]. Severe complications associated with stenting include bleeding and perforation and have been reported in 1.2% of cases[51]. Comparing stent types, migration rates are higher with covered stents than with uncovered ones; in contrast, uncovered stenting has higher obstruction rates[8,52,53]. In addition, some patients may suffer combined obstructive jaundice and GOO. There are several options for treatment, but biliary endoscopic stenting can pose a challenge if a duodenal stent is in place[54]; patients with stent for biliary obstruction who subsequently have an endoscopic enteral stent are at an increased risk of biliary stent dysfunction[55]. Another option is endoscopic double stenting, a combination of biliary and duodenal stent placement, where different approaches could make it possible[56].

Laparoscopic GJ for malignant GOO

Wilson *et al*[57] published the first report of LGJ in two patients with malignant GOO due to advanced HPB cancer. Today, LGJ is a feasible option, and presents improved morbidity and mortality rates compared with the open surgical approach[3].

In 2007, Siddiqui *et al*[58] designed a model for patients with malignant GOO and performed a decision analysis. They concluded that endoscopic enteral stent

Table 1 Systematic review and meta-analysis: Stents *vs* gastrojejunostomy

Ref.	Type of study	GJ studies	Surgery	Endoscopic stent	No differences
Minata *et al*[8], 2016	Systematic review	LGJ (Mehta 2006, Jeurnink 2010) OGJ (Jeurnink 2010, Fiori 2013)	Lower re-intervention rate	Less invasive COVERED: Higher migration UNCOVERED: Higher obstruction	Technical success Complications
Nagaraja *et al*[13], 2014	Meta-analisis	Laparoscopic GJ (Mittal 2004, Mehta 2006, Jeurnink 2007, Jeurnink 2010)		Shorter LOS	Technical and clinical outcomes
Ly *et al*[38], 2010	Systematic review	Open GJ (Jeurnink 2007, El-Shabrawi 2006, Mehta 2006, Espinal 2006, Mejia 2006, del Piano 2005, Maetani 2005, Fiori 2004, Mittal 2004, Maetani 2004, Johnsson 2004, Wong 2002, Yim 2001) Laparoscopic GJ (Jeurnink 2007, Mehta 2006, Mittal 2004)	More major medical complications	More likely to tolerate an oral intake More likely to tolerate an oral diet earlier Shorter LOS	Survival 30 d-mortality Major complications

LOS: Length of stay; GJ: Gastrojejunostomy.

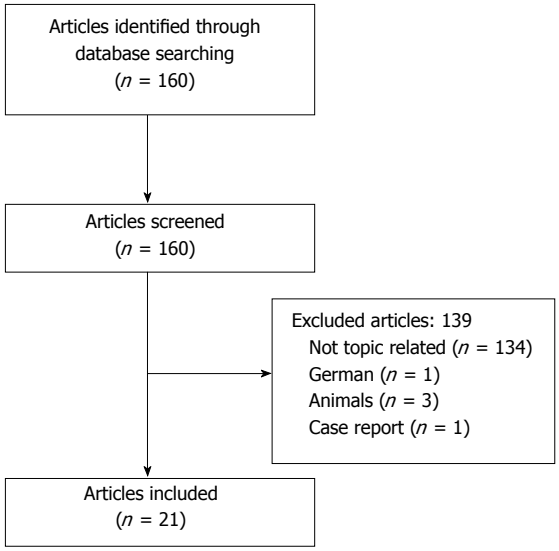

Figure 1 Flowchart.

was a optimal strategy, associated with a 72% success rate and the lowest 1-mo mortality rate (2.1%), one of the drawbacks was recurrent duodenal obstruction, found in up to 25%. They reported a 69% success rate after LGJ (overall 1-mo mortality 2.5% and a cost increase of $10340), and 63% success after open GJ with higher 1-mo mortality (4.5%) and more expensive treatment (a cost increase of $12191)[58].

Given the limited number of controlled trials of the laparoscopic approach in palliative GJ[39,59,60], data available are insufficient to perform an analysis comparing LGJ with OGJ or endoscopic stent[38].

We therefore performed a systematic literature review, in accordance with the PRISMA guidelines, on patients with advanced HPB malignancy who had undergone laparoscopic palliative GJ up to February 2018. The search items were the following MESH terms: [(Gastric outlet obstruction) OR (Gastric Outlet Obstructions) OR (Obstruction, Gastric Outlet) OR (Obstructions, Gastric Outlet) OR (Outlet Obstruction, Gastric) OR (Outlet Obstructions, Gastric) OR (Duodenal obstruction) OR (Duodenal Obstructions) OR (Obstruction, Duodenal) OR (Obstructions, Duodenal)] AND [(Gastric bypass) OR (Bypass, Gastric) OR (Gastrojejunostomy) OR (Gastrojejunostomies) OR (Gastroenterostomy) OR (Gastroenterostomies)] AND [(Laparoscopy) OR (Laparoscopies) OR (Surgical Procedures, Laparoscopic) OR (Laparoscopic Surgical Procedure) OR (Procedure, Laparoscopic Surgical) OR (Procedures, Laparoscopic Surgical) OR (Surgery, Laparoscopic) OR (Laparoscopic Surgical Procedures) OR (Laparoscopic Surgery) OR (Laparoscopic Surgeries) OR (Surgeries, Laparoscopic) OR (Surgical Procedure, Laparoscopic)]. Eligibility criteria were any type of article that included patients with advanced HPB malignancy who had undergone laparoscopic palliative GJ, excluding case reports or reports of prophylactic GJ.

The articles were included or rejected based on the information in the title and summary, and in case of doubt, after reading the complete article.

Figure 1 presents a flowchart of systematic review of patients with advanced HPB malignancy who had undergone laparoscopic palliative GJ. The initial search yielded, 160 articles, but only 21 (13.12%) met the search criteria.

The outcomes and surgical techniques of LGJ for malignant GOO are displayed in Tables 2 and 3[3-5,9,15,37,39,57,60-72]. Most studies were case series (12/21)[5,9,15,57,61-64,66,69,72], five were cohort series[3,4,37,60,68], two case/control studies[70,71] and only two studies were randomized controlled trials (RCT)[39,67]. The studies included different etiologies for GOO, among them benign disease[66] and only nine publications recorded all patients with advanced HPB malignancy[4,5,9,61-65].The systematic review included 495 patients, of whom 55 (11.11%) had advanced HPB cancer and had undergone LGJ. There was a mix of associated treatments for biliary obstruction, including endoscopic stent (ES), percutaneous drainage (PD), and biliary bypass (choledochojejunostomy, CJ; cholecystojejunostomy, CCJ). The results displayed in Table 2, show that there are no standardized outcomes for reporting results after

Table 2 Systematic review of laparoscopic gastrojejunostomy for gastrict obstruction due to advanced hepatobiliary cancer

Ref.	*n*	Type of study	HPB Etiology	Biliary obstruction	Operating time	Perioperative morbidity	Time to initiate intake	Time to solid food	LOS	Duration of food intake	Comment
All HPB Malignancy											
Jeurnink *et al*[60], 2007	95	Cohort: GJ (42) *vs* duodenal stent (53)	GJ: All patients (laparoscopy: 10)	GJ: 17 previous treatment	ND	GJ: 4 major (hemorrhage, severe pain, cholangitis, respiratory failure); 13 minor (mild pain, wound infection, nausea and vomiting)	ND	GJ: 10.1 ± 4.8 d	GJ: 18d (4-55)	ND	
Hamade *et al*[4], 2005	21	Cohort: laparoscopic GJ/CJ/GJ+CJ	All patients	5 biliary bypass, 8 GJ+biliary bypass	gastric bypass 75 min, GJ+CJ 130 min	1 pneumonia, 1 central line sepsis, 1 wound abscess	ND	ND	4 d (1-14)	9 patients untill death	Includes pre-treatment, profilactic and terapeutic GJ
Denley *et al*[9], 2005	18	Case series: LGJ	All patients	ND	ND	2 reconversions, 1 leak, 1 sepsis, 1 DGE	ND	ND	6 (3-22)	15 patients untill death	
Kazanjian *et al*[5], 2004	9	Case series: LGJ	All patients	ND	116 min (75-300)	1 DGE, 1 Cholangitis	ND	4 d (3-6)	7 d (5-18)	ND	4 patient previous stent
Alam *et al*[61], 2003	8	Case series: LGJ	All patients	ND	135 min	Pneumonia (1)	ND	4 (2-7)	7 (5-13)	7 patients untill death	
Kuriansky *et al*[62], 2000	12	Case series: LGJ+biliary bypass	All patients	12 CCJ	89.16 min (35-150)	2 wound infection, 1 pneumonia, 2 DGE, 1 reintervention (bleeding)	ND	ND	6.4 (5-17)	All patients untill death	
Casaccia *et al*[63], 1999	6	Case series: LGJ	All patients	4 ES. 2 Laparoscopic CCJ	82 min (60-135)	1 Bleeding (transfusion)	ND	ND	4.5 (4-6)	ND	
Casaccia *et al*[64], 1998	5	Case series: LGJ	All patients	4 ES. 1 laparoscopic CCJ	ND	1 Bleeding (transfusion)	ND	ND	4 (4-6)	ND	
Rhodes *et al*[65], 1995	16	Case series: laparoscopic CCJ ± GJ (5GJ, 3 both, 9CCJ)	All patients	ND	75 min	1 DGE, 1 ictus	ND	ND	4 d (3-33)	ND	Results of the entire data series
Wilson *et al*[57], 1992	2	Case series: LGJ	All patients	ND	120 min	None	2d	3 d, 4 d	4-5 d	1 patient untill death	
Mixed malignancies											
Zhang *et al*[66], 2011	28	Case series: LGJ for benign/ malignant disease	7 HPB malignancy	ND	170 min	2 reinterventions (anastomotic leak, trocar site hemorrhage), 2 bleeding controlled by endoscopy, 1 ileus, 5 DGE	3d	5 d	8 d (2-83)	ND	Results of the entire data series
Guzman *et al*[3], 2009	20	Cohort: LGJ AND OGJ	Laparoscopy: 8 HPB malignancy	ND	116 min	2 DGE	ND	7 d	8 d	ND	
Navarra *et al*[67], 2006	24	RCT: 12 LGJ *vs* 12 OGJ	Laparoscopy: 5 HPB malignancy	ND	150 min	None	ND	4.08 d	11 d	ND	

Mehta *et al*[39], 2006	27	RCT: 14 LGJ *vs* 13 SEMS	ND	6 patients (ES, PD)	ND	2 bleeding, 1 wound infection, 1 pneumonia, 3 DGE. 3 mortality (sepsis, pneumonia, carcinomatosis)	ND	ND	11.4 D	ND
Al-Rashedy *et al*[68], 2005	26	Cohort: LGJ and OGJ	Laparoscopy: 7 HPB malignancy	ND	ND	2 (13.3%)	ND	ND	3 (3-8)	ND
Khan *et al*[69], 2005	19	Case series: laparoscopic CCJ ± GJ (16 GJ, 1 CCJ, 2 both)	7 HPB malignancy	2 CCJ	164 min single bypass, 245 min double bypass	ND	3d	ND	ND	ND
Mittal *et al*[37], 2004	56	Cohort: 16 OGJ, 14 LGJ, 16 ES.	Laparoscopy: 9 HPB malignancy	None patient	ND	4 pneumonia, 1 ileus, 1 wound infection	ND	5 d (4-8)	13.5 d (6-36) (after procedure 7d)	ND
Bergamaschi *et al*[70], 2002	55	Case/control: antiperistaltic *vs* isoperistaltic LGJ	AP-LGJ: 29 HPB malignancy, IP-LGJ 14 HPB malignancy	ND	100min (AP) *vs* 99min (IP)	14 (II: 1, III: 9, IV: 3)	ND	5.1d (AP) *vs* 5.3 d (IP)	8.4 d (AP) *vs* 8.1 d (IP)	ND
Bergamaschi *et al*[71],1998	22	Case / control: OGJ (prophylactic and GOO treatment) *vs* LGJ (GOO treatment)	Laparoscopy: 9 HPB malignancy	1 ES, 3 PD	94 min	Pneumonia (1), SSI (1), delayed gastric emptying (1)	ND	8.4 (media)	18.4 (media)	ND
Brune *et al*[15], 1997	16	Case series: LGJ	13 HPB malignancy	ES/PD	126 min (70-210)	1 reintervention (hemorrhage), 3 delayed gastric emptying	ND	ND	4.7 (2-8)	16 patients untill death
Nagy *et al*[72], 1995	10	Case series: LGJ	9 HPB malignancy	8 ES/1 PD/ 2 simultaneous CJ	ND	2 reconversions, 1 CCF, 1 pneumonia, 1 CD infection	ND	10 d (4-15)	ND	All patients untill death

HPB: Hepatopancreatic-biliary; LOS: Length of stay; LGJ: Laparoscopic gastrojejunostomy; OGJ: Open gastrojejunostomy; ND: Not described; GOO: Gastric outlet obstruction; CCJ: Cholecystojejunostomy; CJ: Choledochojejunostomy; ES: Endoscopic stent; PD: Percutaneous drainage; AP: Antiperistaltic; IP: Isoperistaltic; CCF: Congestive cardiac failure; RCT: Randomized controlled trial; CD: *Clostridium difficile*; DGE: Delayed gastric emptying.

LGJ. Regarding the surgical technique (Table 3), most LGJ were antecolic-isoperistaltic stapler plus manual suture, but there was no standardized approach for LGJ.

Personal series: palliative laparoscopic gastrojejunostomy

We also performed a retrospective study at the Department of General Surgery and Digestive of the University Hospital of Guadalajara. The period analyzed was January 2009-March 2018. We included all consecutive patients who underwent laparoscopic palliative GJ for malignant GOO due to advanced HPB cancer, excluding prophylactic GJ and OGJ. All patients had histological diagnosis of HPB cancer. For this purpose, the Mambrino XXI® electronic medical history was used.

Our results are shown in Table 4. All GJ were performed by the same surgeon using the same approach (IP, antecolic and stapler plus manual suture). Three patients had previous biliary stent, and another patient needed a percutaneous biliary stent after laparoscopic GJ due to obstructive jaundice. The clinical success rate was 100%, with all patients maintaining oral intake until death. The median time from surgery to hospital discharge was 12 d (range 5-13), excluding hospital stay prior surgery attributable to GOO. One patient died due to sepsis caused by a hepatic abscess on postoperative (PO) day 78, and another died due to carcinomatosis and tumor progression on PO day 82. Median overall-survival was 214.67 d.

Other surgical options for malignant GOO

Several surgical procedures for GJ have been reported since *Devine et al*'s first description in 1925, which introduced a procedure consisting of transection of the stomach and anastomosis between the jejunal loop- and the proximal stump of the stomach[73]. But GJ may be not fully effective due to of DGE or tumor bleeding;

Table 3 Systematic review of laparoscopic gastrojejunostomy for gastrict obstruction due to advanced hepatobiliary cancer: Surgical technique

Ref.	Peristalsis	Location	Type
All HPB malignancy			
Jeurnink *et al*[60], 2007	ND	Antecolic	Completely stapler
Hamade *et al*[4], 2005	IP	Antecolic	Stapler + manual suture
Denley *et al*[9], 2005	IP	Antecolic	Stapler + manual suture
Kazanjian *et al*[5], 2004	ND	Antecolic	Completely stapler
Alam *et al*[61], 2003	IP	ND	Completely stapler
Kuriansky *et al*[62], 2000	ND	Retrocolic	Completely stapler
Casaccia *et al*[63], 1999	ND	Antecolic	Completely stapler/stapler+ manual suture
Casaccia *et al*[64], 1998	ND	Antecolic	Completely stapler/stapler+ manual suture
Rhodes *et al*[65], 1995	ND	ND	Stapler + manual suture
Wilson *et al*[57], 1992	ND	Antecolic	Stapler + manual suture
Mixed malignancies			
Zhang *et al*[66], 2011	ND	Antecolic (majority)	Stapler + manual suture
Guzman *et al*[3], 2009	ND	ND	Stapler + manual suture
Navarra *et al*[67], 2006	IP	Antecolic	Stapler + manual suture
Mehta *et al*[39], 2006	ND	Antecolic	Stapler + manual suture
Al-Rashedy *et al*[68], 2005	ND	Antecolic	Hand-sutured or stapler
Khan *et al*[69], 2005	ND	Antecolic	Stapler + manual suture
Mittal *et al*[37], 2004	ND	ND	ND
Bergamaschi *et al*[70], 2002	29 AP *vs* 14 IP	Antecolic	17 completely stapled/38 stapler+ manual suture
Bergamaschi *et al*[71],1998	ND	ND	7 completely stapled/2 stapler+ manual suture
Brune *et al*[15], 1997	IP	Antecolic	Stapler + manual suture
Nagy *et al*[72], 1995	ND	Antecolic	Stapler + manual suture

IP: Isoperistaltic; AP: Antiperistaltic; ND: Not described.

Table 4 Personal serie of laparoscopic gastrojejunostomy

Age/sex	Biliary obstruction	Surgical technique	Clinical success	Time to initiate intake	Surgery-discharge (d)	90-d morbidity	Duration of food intake	Survival (d)
87/F	No	IP antecolic, stapler + manual suture	Yes	4	12	CD infection	Until death	402
76/M	Biliary stent	IP antecolic, stapler + manual suture	Yes	3	12	No	Until death	228
91/F	No	IP antecolic, stapler + manual suture	Yes	1	5	No	Until death	278
78/F	No	IP antecolic, stapler + manual suture	Yes	3	10	Readmission: Sepsis due to hepatic abscess (death)	78	78
68/F	Biliary stent	IP antecolic, stapler + manual suture	Yes	3	12	Readmission: Intestinal obstruction due to carcinomatosis (death)	82	82
76/M	Biliary stent	IP antecolic, stapler + manual suture	Yes	3	13	Catheter-related bacteriemia. Readmission: Biliary stent due to jaundice.	Until death	220
76/F	No	IP antecolic, stapler + manual suture	Yes	3	5	No	Until death	ND

M: Male; F: Female; IP: Isoperistaltic; CD: *Clostridium difficile*; ND: Not described; LOS: Lenght of stay.

so a modified Devine procedure has been developed, in which the stomach is partially divided into proximal and distal parts, and the proximal part of the stomach is anastomosed to the proximal part of the jejunum[74,75]. This technique, stomach-partitioning GJ (SP-GJ), minimizes contact between food and the tumor and allows endoscopic examination[74]. The first laparoscopic approach for SP-GJ was described by Matsumoto *et al*[76] in 2005. This surgical technique is associated with lower incidence of bleeding and delayed gastric emptying, with no increase in anastomotic leakage[74-78].

Other surgical approaches reported in the literature for the management of malignant GOO include natural orifice transumbilical surgery[79] or a laparoscopic-assisted approach for a circular mechanical GJ, in which the proximal jejunum is exteriorized by laparoscopy via an epigastric trocar-site incision[80].

Novel endoscopic approaches for malignant GOO

EUS-gastroenteroanastomosis (EUS-GE) was first described by Fritscher-Ravens *et al*[81,82] in 2002. It is produced by anatomical puncture from the stomach into the third part of the duodenum (-EUS-guided gastroduodenostomy), or into the jejunum (EUS-guided

Table 5 Technical options for gastric outlet obstruction: advantages and disadvantages

Procedure	Advantages	Disadvantages
Open GJ	Bypass of tumor	Most invasive procedure
	Established surgical procedure	Longer LOS
	Lower re-intervention rate	Nutritional status
	Good long-term results	Critically ill patients
Laparoscopic GJ	Bypass of tumor	Invasive procedure
	Lower re-intervention rate	Longer LOS
	Established surgical procedure	Nutritional status
	Less invasive than open GJ	Critically ill patients
	Good long-term results	
Endoscopic enteral stent	Short procedure time	Stent migration
	Established endoscopic procedure	Patency
	Broad indication regardless patient condition	
	Short LOS	
	Good short-term results	
EUS-GJ	Bypass of tumor	Special device
	Short procedure time	Non-establish endoscopic procedure
	Short LOS	Serious adverse events
	Less invasive	

EUS-GJ: Endoscopic ultrasound gastrojejunostomy; GJ: Gastrojejunostomy; LOS: Length of stay.

gastrojejunostomy)[83].

This new EUS technique involves the placement of a lumen-apposing metal stent (LAMS). Data regarding its use are limited[84-87]. In 2017, Pérez-Miranda *et al*[87] reported the results of a multicenter cohort study comparing EUS-GJ and LGJ. All patients in the EUS-GJ group had symptomatic GOO, compared with only 34% of patients in LGJ group. The clinical success rates in the two groups were 84% *vs* 90%, LOS was 9.4 d *vs* 8.9 d and adverse events were 12% *vs* 41%, with the EUS-GJ group presenting better results in all cases. This is a new EUS technique and it should be reserved for use at experienced centers.

CONCLUSION

Palliative treatment of GOO due to advanced HPB cancer may improve QOL and resolve symptoms. Both a non-operative endoscopic approach and surgical treatment are available (Table 5) and an estimation of probable survival is essential for the choice of treatment. Evaluation of the patient and multidisciplinary expertise are required to select the appropriate approach.

Stent is usually preferred in patients with poor general condition or short life expectancy. LGJ is a feasible, safe and efficient technical option. Given the limited studies and the difficulty of performing prospective controlled trials due to patient heterogeneity, no study can cover all the complexities of malignant GOO and more outcome data are needed. Prospective clinical trials with adequate sample sizes comparing different approaches size are warranted.

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MINIREVIEWS

Mouse models for investigating the underlying mechanisms of nonalcoholic steatohepatitis-derived hepatocellular carcinoma

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Abstract

As the incidence of hepatocellular carcinoma (HCC) caused by infection with the hepatotropic viruses hepatitis B and hepatitis C decreases, greater attention has become focused on HCC caused by nonalcoholic steatohepatitis (NASH), an advanced form of nonalcoholic fatty liver disease which has shown increasing prevalence in correspondence with the overall increase in metabolic syndrome over the recent decades. Several clinical population studies have shown a positive relationship between NASH and HCC, while also providing initial insights into the underlying mechanisms of HCC development from NASH. Research into the pathological progression of NASH to HCC has advanced by use of several beneficial rodent models. In this review, we summarize the established mouse models for preclinical research of NASH-associated HCC and discuss the underlying hepatic mechanisms of NASH-related tumorigenesis identified to date that could lead to new targets for treatment and prevention.

Key words: Hepatocellular carcinoma; Nonalcoholic steatohepatitis; Nonalcoholic fatty liver disease

Core tip: This review provides a brief overview of the molecular mechanisms underlying progression

to hepatocellular carcinoma from nonalcoholic steatohepatitis that have been identified to date using the array of mouse models currently available and popular in the experimental field.

Takakura K, Oikawa T, Tomita Y, Mizuno Y, Nakano M, Saeki C, Torisu Y, Saruta M. Mouse models for investigating the underlying mechanisms of nonalcoholic steatohepatitis-derived hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(18): 1989-1994 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1989.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1989

INTRODUCTION

As Western diet and problems with food satiation have spread across the globe in recent years, there has been a concomitant increase in patients with nonalcoholic fatty liver disease (NAFLD) and its progressive form of nonalcoholic steatohepatitis (NASH). This increase is the result of prevailing metabolic syndrome, including obesity, diabetes and hyperlipidemia[1-4]. The distinctive characteristic of NAFLD is its diversity of conditions, from simple fatty accumulation in the liver to hepatic injury and inflammation with or without fibrosis[2,5-7]. The sequential progression to NASH puts the sufferer at risk for irreversible liver cirrhosis and hepatocellular carcinoma (HCC)[4,7], causing the patient to require more medical attention due to the increased morbidity and mortality[8]. Indeed, HCC is a leading indication for liver transplantation, especially in developed countries[9,10].

Compared with the long history of both clinical and laboratory investigations to elucidate the molecular pathogenesis of HCC derived from chronic hepatotropic virus infections, particularly with hepatitis B virus and hepatitis C virus, and from alcoholic liver disease, the pathologic mechanisms of NASH-associated HCC (NASH-HCC) remain largely uninvestigated and unknown. The public health threat associated with the increasing incidence of NASH-HCC[11], however, highlights the urgent need to gain a more comprehensive and detailed understanding of the mechanisms which mediate NASH-HCC progression. Several experimental mouse models exist for such studies[12-15] and should be continuously applied to preclinical investigations into the pathogenic pathways of NASH-HCC to advance the subsequent development of methods to manage the modern increasing clinical trend.

Here, we summarize the established mouse models for preclinical research of NASH-HCC progression (Table 1) and discuss the revealed mechanisms and the future prospective of NASH-related tumorigenesis in liver which could lead to new targets for treatment or prevention (Figure 1). Of note, we recognize the existence of other available rodent models which can also be used for assessing the mechanisms of NASH-HCC; however, we focused this review on the ones which are most representative of metabolic syndrome-associated steatohepatitis and which generate HCC unfailingly from NASH status within a certain period of time.

CONFIRMED TUMORIGENIC MECHANISMS OF CURRENT NASH-HCC MOUSE MODELS

The established mouse models for preclinical research of NASH-HCC progression are listed below (Table 1).

PTEN null mice

PTEN, a tumor suppressor gene which antagonizes the PI3K/Akt pathway, is mutated in many human cancers, including HCC, and is essential for maintaining homeostasis and preventing oncogenesis in the liver. Decreased *Pten* expression leads to increased tumor grade, advanced stage and poor prognosis. Hepatocyte-specific *Pten* null mice were generated by Horie *et al*[12], wherein steatohepatitis emerges at 10 wk old and hepatic tumors at 40-44 wk old. The liver tumors become adenomas in 100% of these mice or HCC in 66% at 74-78 wk old, due to the *Pten* deficiency (*Pten* knock-out, KO) causing lipid accumulation in hepatocytes. In general, these mice have revealed that *Pten* function is crucial for preventing tumorigenesis in liver.

Several other research groups have uncovered different mechanisms of NASH-HCC by using the *Pten* null mouse model. For example, a study of eicosapentaenoic acid (EPA; a typical dietary n-3 polyunsaturated fatty acid contained in fish oil and a reagent for upgrading lipid metabolism[16]) performed by Ishii *et al*[17] showed the effect of EPA on steatohepatitis and tumor formation in *Pten* null mice. The data confirmed that the steatotic change, accumulation of inflammatory cells and presence of ballooning hepatocytes were significantly decreased in the EPA group compared with the control group. In addition, liver adenomas developed in 63% of the control group mice, as compared with 0% of the EPA group mice, by 40 wk of age. HCC developed in 75% of the control group and 13% of the EPA group of the *Pten* KO mice at 76 wk old. In addition, MAPK and Akt, which are both downstream signaling molecules of Ras, were found to be activated in hepatocytes of the *Pten* KO mice, thereby promoting tumorigenesis[18]. Collectively, these data suggested that EPA alters fatty acid composition in liver and suppresses the development of HCC by inactivating these signaling pathways in *Pten* null mice.

In another study of the *Pten* null mice, reduction of glucose-regulated protein 78 (GRP78; a molecular chaperone elevated in several human cancers, including HCC[19,20], and which is critical for endoplasmic reticulum folding, stress signaling and PI3K/Akt activation) promoted liver steatosis and liver injury at

Table 1 Mouse models of nonalcoholic steatohepatitis-associated hepatocellular carcinoma

List	Backgrounds	Inducer of NASH/HCC	Carcinogenic duration	HCC occurrence (%)	Ref.
PTEN null mice	Genetic	Spontaneous	40 wk	66 (74-78 wk)	[12,17,18,21,22]
MC4R KO mice	Genetic	HFC diet	1 yr	100	[13,29,31]
STAM mice	DM/HL	Streptozotocin, HFC diet	20 wk	100	[14,32-36]
ALR KO mice	Genetic	Spontaneous	1 yr	60	[15]

HFC: High fat/calorie; DM: Diabetes; HL: Hyperlipidemia; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

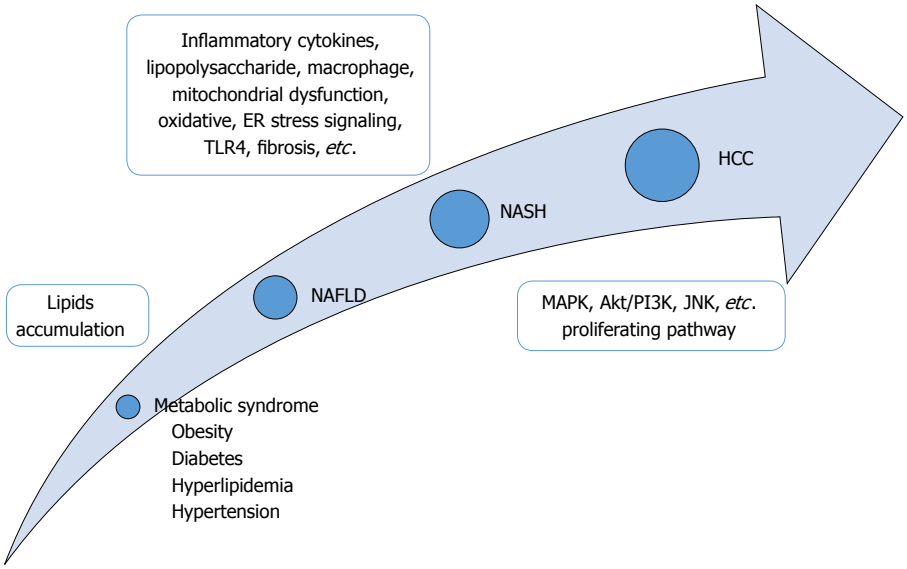

Figure 1 Developmental process of hepatocellular carcinoma *via* nonalcoholic steatohepatitis. Based on excessive lipids accumulation, several factors such as inflammatory cytokines, oxidative stress or proliferating pathways are involved in the whole process of hepatocellular carcinoma development from nonalcoholic steatohepatitis status *via* nonalcoholic fatty liver disease. NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

3 mo of age and liver tumors at 6 mo of age[21]. These effects proceeded HCC or cholangiocarcinoma, which developed at 8-9 mo of age and was accompanied by elevation of p-JNK; in contrast, the GRP78 normal *Pten* null mice never generated tumor lesions in liver, as assessed out to 14 mo of age[21]. Collectively, these data suggested that JNK might contribute to acceleration of tumorigenesis in liver. Accordingly, these data demonstrated GPR78 as a regulator for *Pten* loss-mediated liver steatosis and tumor progression on the basis of p-JNK elevation.

In a third study of the *Pten* null nice, Miura *et al*[22] showed that liver tumors emerged after 36 wk of age, although no liver tumors were found in *Pten* normal mice until 72 wk of age. Toll-like receptor (TLR) 4 expressed on macrophages was found to contribute to the development of steatohepatitis and HCC in *Pten* KO mice. In general, gut-derived materials stimulate the immune system, including the TLRs which recognize bacterial components. TLR4, in particular, senses components of Gram-negative bacteria, including the lipopolysaccharide (LPS)[23]. In this way, TLRs affect the development of liver diseases. Moreover, macrophages are known to be a major source of proinflammatory cytokines which facilitate the progression of steatohepatitis[24,25] and Ly6C is a marker for inflammatory macrophages[26]. Hepatic macrophages isolated from the *Pten* null mice showed an increased expression of Ly6C. In addition, TLR4 signaling was shown to promote hepatic inflammation as well as subsequent liver tumor growth in the *Pten* null mice. Antibiotic treatment suppressed the tumor growth, in concert with a decreasing LPS level in the portal vein, suggesting that the gut microbiota serves as a source of TLR4 ligand(s) and that the Ly6C-positive macrophages play a role in tumor development in *Pten* null mice. Collectively, these data indicate that gut-derived LPS-induced inflammation *via* TLR4 on macrophages and TLR4-mediated inflammation result in HCC.

Melanocortin 4 receptor KO mice

Melanocortin 4 receptor (MC4R), a seven-transmembrane G protein-coupled receptor, is involved in regulation of body weight; hence, *MC4R* gene mutation is the major monogenic origin of obesity in human[27,28]. Feeding of a high-fat diet to MC4R-deficient (MC4R-KO) mice for 20 wk and 1 year leads to NASH and multiple well-differentiated HCC formations in the liver, respectively[13]. Similar to the findings in *Pten* null mice, Konuma *et al*[29]

found that highly-purified EPA treatment of MC4R-KO mice effectively inhibited the development of liver fibrosis without affecting body weight.

According to their previous study, hepatic crown-like structures (hCLSs), a unique histological feature, were found to play a pivotal role in the progression from simple steatosis to NASH[30], with EPA markedly suppressing hCLS formation and fibrosis *via* prevention of hepatocyte injury. Thus, it was concluded that the beneficial effect of EPA involved the hCLSs. In addition, canagliflozin (CANA, a sodium glucose cotransporter 2 inhibitor and antidiabetic drug) was shown to attenuate NASH-HCC in another study[31]. Based on the evidence that CANA induces adipose expansion without promoting macrophage augmentation, inflammation or fibrosis and altered glutathione metabolism to reduce oxidative stress in adipose tissue, the authors concluded that the decreased hepatic fat accumulation upon CANA treatment suppresses hepatic inflammation, fibrosis at 20 wk and subsequent NASH-HCC at 52 wk in Western diet-fed MC4R-KO mice.

STAM mice

The STAM mouse model was generated by neonatal male C57BL/6J mice exposure to low-dose streptozotocin at 2 d after birth followed by feeding with a high-fat diet after 4 wk of age[14]. As a result, NASH developed at 8 wk and HCC at 16-20 wk. This mouse model has specific positive features, such as the average duration of HCC occurrence being within 16-20 wk of age, the number of HCC nodules being over 4 in any single mouse, the basal liver function being relatively preserved and there being no visible metastasis in the entire body[32]. Moreover, this model has the substantial benefit of its HCC development from NASH being identical to the known progression in human patients, but with the whole process being completed within a relatively short period of time.

By using the STAM model, four studies have uncovered several of the mechanisms underlying NASH-HCC. First, Lau *et al*[33] demonstrated that cancer-associated fibroblasts, which regulate liver tumor-initiating cells, are augmented in parallel with increasing human growth factor (HGF) level during fibrosis and that HGF-induced FRA1 activation is related to fibrosis-dependent HCC development. These data suggest that cancer-associated fibroblast-derived, HGF-mediated FRA1 can be a new therapeutic target for NASH-HCC. Second, Fernandes *et al*[34] showed that solithromycin, a novel macrolide antibiotic, suppressed NASH, fibrosis and NASH-HCC by modulating the gluconeogenesis pathway, in particular the components of fructose 1, 6-biphosphatase and glucose-6-phosphatase which are regulated by protein kinase C epsilon. Solithromycin improved the hepatic morphological features, such as the hepatocyte ballooning degeneration, and functions, as evidenced by reduction in NAFLD activity score along with decreased inflammation, fibrosis and HCC progression. This mechanism was ultimately suggested as a candidate factor of novel treatment of NASH-HCC.

Third, Conti *et al*[35] revealed that aberrant expression of hepatic micro (mi)RNAs, such as miR-34a-5p, miR-93-5p, miR-221-3p and miR-222-3p, indicates their mechanistic significance in NASH-HCC tumorigenesis; specifically, 10 over-expressed miRNAs were identified. It is well known that human HCC tumorigenesis is associated with extensive genomic alterations. Therefore, the authors concluded that the altered expression profile of these miRNAs could be a surrogate marker for the initiation and progression of NASH-HCC.

Finally, based on the confirmed finding that NASH-HCC is associated with metabolic alterations in hepatic lipid homeostasis, Pogribny *et al*[36] indicated that one of the specific features of NASH-HCC is a significant dysregulation of 1-carbon homeostasis, with decreased expression of key 1-carbon metabolism genes, especially of the S-adenosylhomocysteine hydrolase (*Ahcy*) gene, and increased expression of the S-adenosyl-L-homocysteine (*SAH*) gene. Their results suggest that the inhibition of *Ahcy* expression may be a trigger of SAH elevation and subsequent progression of NASH-HCC.

Augmenter of liver regeneration-KO mice

Augmenter of liver regeneration (ALR), a hepatic growth factor, is widely known as a pleiotropic protein. ALR is critical for mitochondrial function, lipid homeostasis and cell survival. Gandhi *et al*[15] generated a liver-specific ALR-L-KO mouse and reported that depletion of hepatic ALR caused steatosis, mitochondrial degeneration and apoptosis of hepatocytes at 2 wk of age. These effects were followed by consecutive cell death, sustained inflammation at 4 wk, fibrosis/cirrhosis at 8 wk and eventually HCC formation (in 60%) at 1 year. Thus, it was theorized that inhibition of ALR synthesis in hepatocytes could lead to mitochondrial dysfunction and cell death, resulting in consecutive NASH and HCC occurrence.

FUTURE PERSPECTIVES FOR THE STUDY OF NASH-HCC BY ANIMAL MODELS

The "two-hit" hypothesis of the underlying mechanism of NASH-HCC involves the excessive accumulation of lipids in liver as the first step, thereby promoting sensitization to LPS, oxidative stress and inflammatory cytokines, representing the second hit[37-39] (Figure 1). Recently, Tilg and Moschen[40] proposed a "multiple-hit" hypothesis, in which various factors derived from gut and adipose tissue might take place in parallel during the progression from NAFLD to NASH. However, the definitive mechanisms in the progression from simple fatty liver to NASH and HCC are still under investigation, due to the inherent complexity of the functional combination of several factors. For some time, it was believed that the lack of appropriate animal models which were able to sufficiently reflect the actual

process of human NASH-HCC progression was the main obstacle to such research[41]. In recent years, however, the situation has changed according to the development and availability of several rodent models. Each model harbors different specific characteristics, including genetic background, obesity status, diet induction, *etc*. Thus, researchers can now evaluate the mechanisms of NASH-HCC related to a specific factor/parameter by using these animal models.

According to the overall analyses of hepatocarcinogenesis in each of the mouse models discussed above, it is the STAM mice that generate HCC unfailingly and most rapidly. The considerable demerit of this mouse model, however, is the obscurity of the original gene of tumorigenesis for HCC due to lack of genetic manipulation and the inclusion of diabetes and hyperlipidemia in the background. Genetic manipulation in mouse models, such as of the PTEN-KO or ALR-KO, is a useful means by which to clarify the role of a specific gene in the molecular foundation of NASH-HCC progression; although, the sequential progression to HCC in these models has a relatively long duration and HCC occurrence is uncertain.

It is still questionable whether or not these available mouse models represent the initiating and/or progression processes of *bona fide* human NASH-HCC. Furthermore, it is noteworthy that among actual NASH patients there are individual differences in degree of fibrosis and timing of tumorigenesis in liver. At the present time, however, it is undoubted that these mouse models are essential for investigating the underlying mechanisms of NASH-HCC. Therefore, the future research targets may move forward towards gaining a more comprehensive NASH-HCC evaluation by using these mouse models.

CONCLUSION

Several mouse models have become available in recent years that support investigation into the underlying mechanisms of NASH-HCC. In response to the growing demand for better management of NASH-HCC, further inquiries are expected by researchers upon selecting an appropriate NASH mouse model according to the specific mechanisms and/or therapeutic targets of interest. After that, we hope to get some breakthrough for new treatment or prevention of NASH-HCC in the near future.

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ORIGINAL ARTICLE

Basic Study

Microbiota modification by probiotic supplementation reduces colitis associated colon cancer in mice

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Abstract

AIM

To investigate the effect of probiotic supplementation during the development of an experimental model of colitis associated colon cancer (CAC).

METHODS

C57BL/6 mice received an intraperitoneal injection of azoxymethane (10 mg/kg), followed by three cycles of sodium dextran sulphate diluted in water (5% w/v). Probiotic group received daily a mixture of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*. Microbiota composition was assessed by 16S

rRNA Illumina HiSeq sequencing. Colon samples were collected for histological analysis. Tumor cytokines was assessed by Real Time-PCR (Polymerase Chain Reaction); and serum cytokines by Multiplex assay. All tests were two-sided. The level of significance was set at $P < 0.05$. Graphs were generated and statistical analysis performed using the software GraphPad Prism 5.0. The project was approved by the institutional review board committee.

RESULTS

At day 60 after azoxymethane injection, the mean number of tumours in the probiotic group was 40% lower than that in the control group, and the probiotic group exhibited tumours of smaller size (< 2 mm) ($P < 0.05$). There was no difference in richness and diversity between groups. However, there was a significant difference in beta diversity in the multidimensional scaling analysis. The abundance of the genera *Lactobacillus*, *Bifidobacterium*, *Allobaculum*, *Clostridium* XI and *Clostridium* XVIII increased in the probiotic group ($P < 0.05$). The microbial change was accompanied by reduced colitis, demonstrated by a 46% reduction in the colon inflammatory index; reduced expression of the serum chemokines RANTES and Eotaxin; decreased p-IKK and TNF-α and increased IL-10 expression in the colon.

CONCLUSION

Our results suggest a potential chemopreventive effect of probiotic on CAC. Probiotic supplementation changes microbiota structure and regulates the inflammatory response, reducing colitis and preventing CAC.

Key words: Intestinal microbiota; Chemoprevention; *Lactobacillus acidophilus*; *Lactobacillus rhamnosus*; *Bifidobacterium bifidum*

Core tip: Intestinal microbiota has an essential role in carcinogenesis, acting in promotion of inflammation, proliferation and neoplastic progression. Probiotic supplementation is an alternative means of favourably modulating the intestinal microbiota. In this study, we investigate the effect of supplementation with a *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* mixture during the development of an experimental model of colitis-associated colon cancer. Probiotic supplementation on colorectal cancer changed the microbiota and reduced inflammation in the colon, probably by regulating the inflammatory response, and reducing inflammatory cell infiltration by lowering chemokine expression, thus preventing colitis.

Mendes MC, Paulino DS, Brambilla SR, Camargo JA, Persinoti GF, Carvalheira JB. Microbiota modification by probiotic supplementation reduces colitis associated colon cancer in mice. *World J Gastroenterol* 2018; 24(18): 1995-2008 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1995.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1995

INTRODUCTION

Colon cancer involves a complex and heterogeneous mechanism, mostly induced by the accumulation of somatic mutations over time, which are caused by environmental factors, diet, microbial exposure and metabolites and the host immune response[1]. Although the causes of colon cancer are not well established, inflammation has been implicated from initiation to promotion of the disease, even for those tumours that do not have a direct causal relationship to inflammation[2]. Tumour-promoting inflammation is a hallmark of cancer, and there is strong evidence that inflammation plays a critical role in cancer development[3]. The balance between the expression of mediators and immunological modulators, as well as the amount and activation of different types of inflammatory cells in the tumour microenvironment, will determine the tumour growth rate[4].

The intestinal microbiota may act as a link between colon cancer-promoting factors and the stages of carcinogenesis[5]. Alteration in microbial composition and diversity is considered essential for the promotion of inflammation, proliferation and neoplastic progression[6]. Studies evaluating the composition of the microbiota in colorectal cancer (CRC) identified that bacteria such as *Bacteroides*, *Parabacteroides*, *Alistipes*, *Akkermansia* spp. *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae* and *Methanobacteriales* are commonly increased. Others, such as *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium* spp., *Roseburia* and *Treponema,* are consistently decreased[6]. However, these association studies cannot determine if this diversity is a cause or a consequence of CRC. As a result, methods that can selectively manipulate the microbiota have emerged as a strategy that may aid in the prevention of cancer.

The intestinal microbiota can be modulated by several factors such as environment, radiation, surgery, medicines, aging, diet, lifestyle and host genetic. Not coincidentally, these factors are also related to inflammation and colon cancer risk[7-9]. Another important way of modulating the intestinal microbiota is through the supplementation of bacterial strains (probiotics). Probiotic supplements are monoassociated cultures or a mix of living microorganisms; *Lactobacillus rhamnosus, Lactobacillus reuteri, L. acidophilus, Lactobacillus bulgaricus, Bifidobacterium infantis, Bifidobacterium bifidum* and *Saccharomyces boulardii* are commonly used as probiotics[10]. Probiotic strains are usually found in dairy products such as yogurts and cheeses or used as food supplements or drugs.

The beneficial effects of probiotic bacteria were recognized more than 100 years ago by Metchnikoff[11]. Modifications to the microbial community can prevent or treat various gastrointestinal disorders such as inflammatory bowel disease and irritable bowel syndrome[12], as well as systemic diseases such as eczema[13], respiratory infections[14], asthma[15] and diabetes[16].

Mechanistically, probiotics may reduce cancer risk by exerting several effects, including destruction of potential carcinogens, reducing microbial genotoxicity, altering the metabolites produced by the microbiota, producing anti-tumourigenic and anti-mutagenic compounds, competing with pathogenic bacteria, increasing the intestinal barrier, increasing the innate immune response of the host and modulating cell proliferation and anti-apoptotic pathways[17-21].

Although the effects of probiotics have been investigated in *in vitro* experiments, animal models, and some human gastrointestinal diseases, little is known about the interaction between probiotic supplementation, changes in the intestinal microbiota and neoplastic transformations of the gastrointestinal mucosa[19,22].

Thus, the aim of this work is to investigate the effect of supplementation of a *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* mixture on the intestinal microbiota, inflammation and neoplastic alterations in the gastrointestinal mucosa during the development of an experimental model of colitis associated colon cancer.

MATERIALS AND METHODS

Animals

Eight-week-old, male C57BL/6 mice weighing approximately 25 g were provided by the central laboratory of the State University of Campinas (UNICAMP) (Campinas, SP, Brazil). All experiments were conducted in order to minimize the pain and discomfort of the animals. Animals were maintained in cages with a maximum of 5 animals, with free access to water and food, in a bed of wood shavings, controlled temperature by air-conditioned, in a light-dark cycle of 12 h. Intragastric gavage administration was done carefully, with the animal immobilized, using gavage needle appropriate for mice. All procedures were performed according to the Ethical Principles in Animal Experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL), with the current law n° 11.794 of October 8, 2008 and the decree n° 6.899 of July 15, 2009. The Ethics Committee on Animal Use (CEUA) of UNICAMP approved the project, according to protocol no. 2761-1.

Experimental design

Animals were randomly divided into two experimental groups (control and probiotic), both of which received a standard diet (AIN93-M). The probiotic group received by gavage daily 0.6 billion CFU (colony forming units) each of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*, diluted in 200 μL of drinking water, while the control group received 200 μL of drinking water daily. Treatment starts one week before colon cancer induction and finished one day before animal sacrifice. Each group started with 15 animals, which were identified and monitored individually throughout the experiment, and their weights were evaluated weekly. Colon cancer induction was done by intraperitoneally injection of 10 mg/kg of Azoxymethane (Sigma-Aldrich®). After 1 wk, 2.5% dextran sulphate sodium (DSS, MW 36-50 kDa) (MP Biomedical, Inc) was supplied in the drinking water for 5 d, followed by 14 d with unsupplemented drinking water. This cycle was repeated two additional times, and the mice were sacrificed 10 d after the last cycle, according Greten *et al*[23]. Tumour samples were collected for RT-PCR and cytokine analysis. Colon tissues were collected for western blotting. Colon faeces were collected for 16S rRNA sequencing. Blood was collected to obtain serum for analysis of serum cytokines. Tumour and colon samples were collected and frozen immediately in liquid nitrogen and all samples were stored at -80 ℃ until analysis.

Microbiota analysis by 16S rRNA sequencing

DNA was extracted from faecal material using a QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) and 50 ng were used for cDNA library synthesis with the Rapid Library Preparation Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. The cDNA was analysed with a Bioanalyzer and High Sensitive DNA Kit (Agilent Technologies Inc., Santa Clara, CA, United States) to ensure equimolar use of the samples in PCR. These samples were then sequenced with a 16S Metagenomic Sequencing® Illumina Kit combined with the HiSeq 2500 System (Illumina) sequencer, according to the manufacturer's instructions. Sequence reads obtained from the V4 region of the 16S gene were analysed according to the UPARSE pipeline[24], using the USEARCH v9.2.64 package. For OTU clustering a threshold of 97% similarity was used through the UPARSE-OTU algorithm. α- and β-diversity analyses were calculated using the R package Phyloseq v.1.19.1[25] and vegan 2.4_2 packets, using the OTU table normalized to the smallest sample size. Taxa with differential abundance between groups were identified using the Kruskal-Wallis test ($P < 0.05$). In the bar plot are shown those taxa with relative abundance greater than 1%.

Cytokines analysis

Analysis of cytokines in serum and tumour tissue was performed by multiplex immunoassay (Bio Plex Pro Mouse Cytokine 23 Plex Panel - Bio Rad, Code: M60009RDPD) according to the manufacturer's instructions. Tumor tissue protein were extracted previously with appropriate protein extraction buffer, in TissueLyser equipment for 3 min at 30 rpm. After 20 min of rest, the samples were centrifuged at 11000 rpm for 30 min at 4 ℃. The supernatant was collected and used for analysis after protein quantification.

Real-time PCR

Tumour tissue was extracted using the RNeasy Mini Kit

(Qiagen®) on the QIAcube (Qiagen®), according to the manufacturer's recommendations. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, United States) at a wavelength of 260 nm. cDNA was synthesized following the recommendations of the cDNA Synthesis Kit (Thermo-Scientific). Real-time PCR reactions were performed using the TaqMan ™ system (Applied Biosystems), which consists of a pair of primers and a fluorophore- labelled probe. The cycling conditions used were: 50 ℃ for 2 min, 95 ℃ for 10 min and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The relative gene expression values were obtained by analyzing the results in the Applied Biosystems 7500 System SDS Software program. Expression levels of the genes of interest were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh; Mm99999915_g1TaqMan®). The genes of interest studied were IL-6 (Mm00446190_m1TaqMan®), IL-1β (Mm00434228_m1 TaqMan®), TNF (Mm00443258_m1 TaqMan®), IL-10 (Mm01288386_m1 TaqMan®), IL-4 (Mm00445259_m1TaqMan®), IL-13 (Mm00434204_m1 TaqMan®) and Tgfβ1 (TaqMan Mm01178820_m1).

Inflammatory index

Colons and tumours were removed for histology. Tissues were processed and fixed on microscopic slides and stained with haematoxylin and eosin. The inflammatory index includes verification of the severity of the areas of epithelial degeneration, focal or multifocal areas, erosions of the epithelium, presence of ulcers, tissue hyperplasia and size of the affected area. Analysis of the inflammatory index was performed according to Cooper *et al*[26].

Western blotting

Colon tissue was extracted using a protein extraction buffer (1% Triton X-100, 100 mmol/L Tris (pH 7.4), 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenylmethanesulfonyl fluoride and 0.1 mg/mL aprotinin), Laemmli sample buffer containing 100 mmol/L DTT was added and the mixture was heated to 100 ℃ for 5 min (ref). For total extracts, similar-sized aliquots were subjected to 8%-15% SDS-PAGE. The samples were electrophoresed for the separation of the proteins, being labelled with a marker of known molecular weight (Thermo Scientific PageRuler Prestained Protein Ladder). Using a wet transfer apparatus, the resolved proteins were blotted onto nitrocellulose membranes (Bio-Rad). Then, membranes were blocked with 5% milk solution and incubated overnight at 4 ℃ with specific antibodies. The antibodies used were anti-phospho-IKK Ser180/Ser181 (Santa Cruz Biotechnology, SC-23470-R), anti-IKK alpha (Santa Cruz Biotechnology, SC-7183), anti-TNF (Cell Signaling Technology, Cell-3707), anti-IL10 (Cell Signaling Technology, Cell-12163) and anti-β-Tubulin (Cell Signaling Technology, Cell-2146). Bands of interest were distinguished according to the protein ladder molecular weight. These membranes were exposed to a chemiluminescence solution (SuperSignal West Pico Chemiluminescent Substrate (Pierce)) and band intensities were revealed by optical densitometry of developed autoradiographs or in a ChemiDoc MP (Bio-Rad).

Statistical analysis

Results were expressed as mean ± SD. The primary outcome was number of tumors. Intestinal microbiota abundance and diversity, inflammatory index and cytokines expression were the secondary outcomes. The Mann-Whitney *U* test was used for comparisons between two groups for continuous variables and Chi-square test was used to compare categorical variables. All tests were two-sided. The level of significance was set at $P < 0.05$. Graphs were generated and statistical analysis performed using the software GraphPad Prism 5.0. Statistical analysis for microbiota data were described in "Microbiota analysis by 16S rRNA sequencing" section.

RESULTS

Probiotic supplementation reduces tumour incidence in a colitis associated colorectal model

Using a CRC model associated with colitis, we investigated the role of probiotic supplementation in the development of CRC. To this end, mice received an intraperitoneal injection of azoxymethane, followed by three cycles of treatment with sodium dextran sulphate (DSS) diluted in water. Two hundred microliters of water or probiotics (0.6×10^9 *L. acidophilus*, 0.6×10^9 *L. rhamnosus* and 0.6×10^9 *B. bifidum*) was provided daily by gavage. The number of tumours was quantified at day 60 after azoxymethane injection. As shown in Figure 1A, the mean number of tumours was 9.7 (± 5.7) (n = 33) in the control group and 5.8 (± 3.3) (n = 29) in the probiotic group, which represents a 40% reduction (P = 0.001). There was no difference in mean tumour size between the groups [control = 3.5 cm (± 1.4) (n = 32), probiotic = 3.0 cm (± 1.7) (n = 29), P = 0.14]; however, the probiotic group presented more tumours of smaller size (< 2 mm) (P = 0.0002) (Figure 1B). These results are represented in the images in Figure 1C. There was no statistically significant difference between initial and final mean weights [control = 1.5 (± 2.7) (n = 31), probiotic = 0.3 (± 3.7) (n = 31), P = 0.21] (Figure 1D).

Probiotic supplementation changes the gut microbiota in the colon

Next, we investigated how probiotic strain supplementation interfered with the abundance and diversity of the intestinal microbiota in the colon and cecum of the probiotic group compared with the control group. Faecal samples from the cecum and colon were collected on day 60 and immediately frozen until the date of DNA extraction. The microbiota profile was

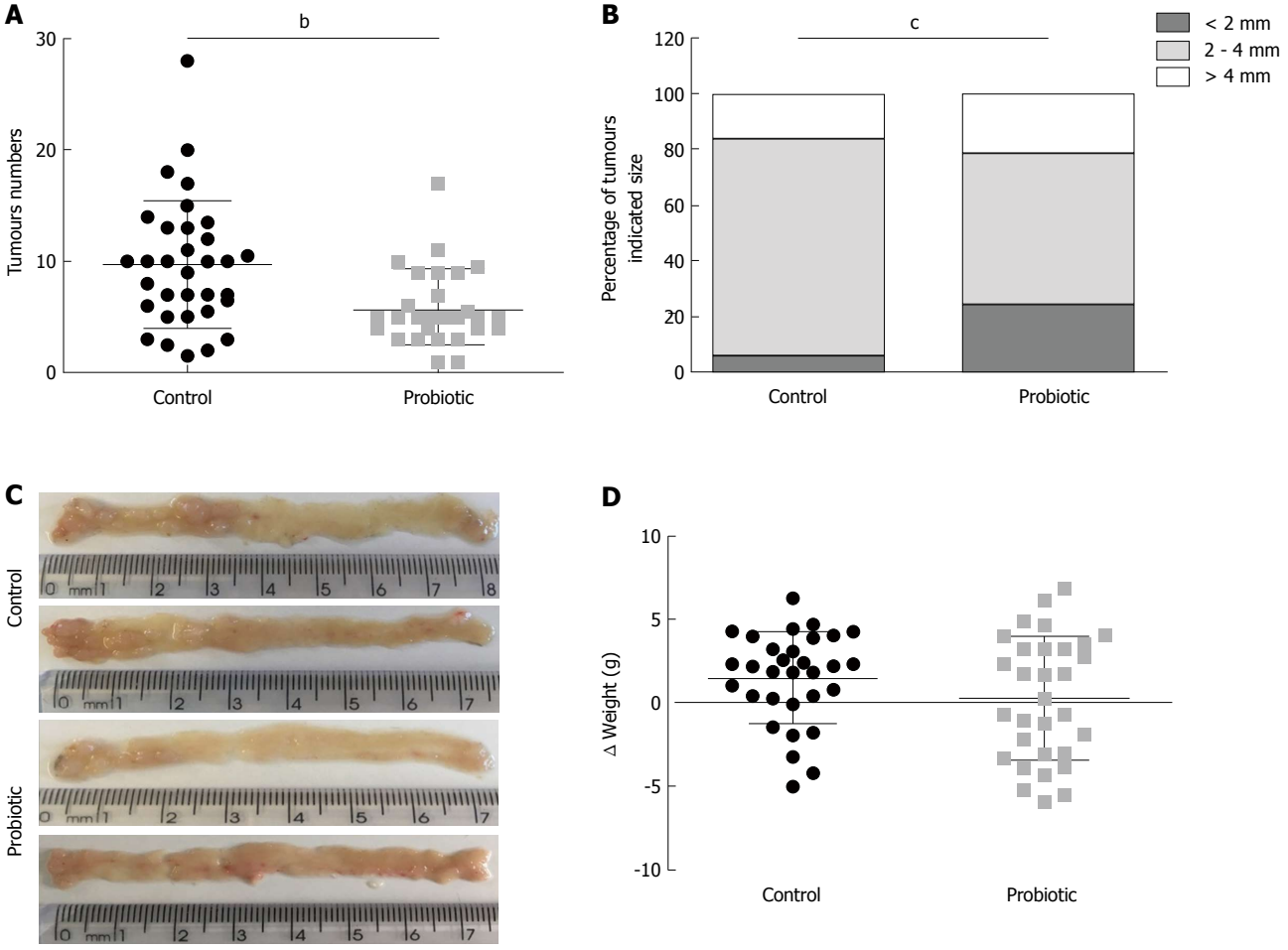

Figure 1 Probiotic supplementation reduces tumour incidence in a colorectal cancer model associated with colitis. Number (A) and size (B) of colon tumours in the control (n = 33) and probiotic (n = 29) groups. C: Representative images of tumours in the colons of the control and probiotic groups at day 60 after injection with azoxymethane. D: Change in body weight during treatment with azoxymethane and DSS in the control (n = 31) and probiotic (n = 31) groups. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; Mann-whitney U test (A and D), Chi-square test (B). Data are from three independent experiments and are presented as mean and standard deviation.

characterized by 16S rRNA Illumina HiSeq sequencing. Our results indicate that probiotic supplementation did not change the alpha diversity of the intestinal microbiota. Comparisons of species richness by total number of operational taxonomic units and Chao1 index did not reveal differences between groups; neither were there differences in diversity assessed by Shannon index (Figure 2A-C). However, multidimensional ordering analysis showed a difference in beta diversity between the control and probiotic groups; in this analysis a closer proximity between points indicated a higher similarity between samples (Figure 2D). It is possible to distinguish two fields in the plot, probiotic in the lower right quadrant and control in the upper left quadrant. There was a significant difference between the control and probiotic groups according to Permanova analysis ($P < 0.001$). Rarefaction curves and the Richness diversity index are shown in the supplemental material (Supplementary Figure 1). Moreover, probiotic supplementation modulated the intestinal microbiota in the colon at the phylum level, generating an increase in bacteria of the phylum Actinobacteria ($P < 0.05$) (Figure 2E and F). In addition, in the taxonomic analysis we highlight the statistically significant difference found at the genus level of *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Allobaculum*, *Clostridium* XI and *Clostridium* XVIII, which were increased in the probiotic group, while *Clostridium* XIVa was reduced in probiotic group (Figure 3A and B); other genera with a statistically significant difference between groups are shown in the supplemental material (Supplementary Figure 2). Taxonomy plots on the class, order and family levels are shown in the supplemental material (Supplementary Figure 3).

Probiotic supplementation modulates inflammation in a colitis associated colorectal carcinogenesis model

At day 60, the colonic tissue were extracted and prepared for histological analysis, and tissue inflammation was assessed by determining the inflammatory index. The probiotic group had a lower inflammatory index than the control group [control = 7.9 (± 1.6), n = 9, probiotic = 4.2 (± 1.0), n = 9; P = 0.0005], an approximately 46% reduction (Figure 4A and B). Spleen weight did not differ significantly between treatments [control = 0.18 (± 0.05), n = 21; probiotic = 0.12 (± 0.06), n = 25; P =

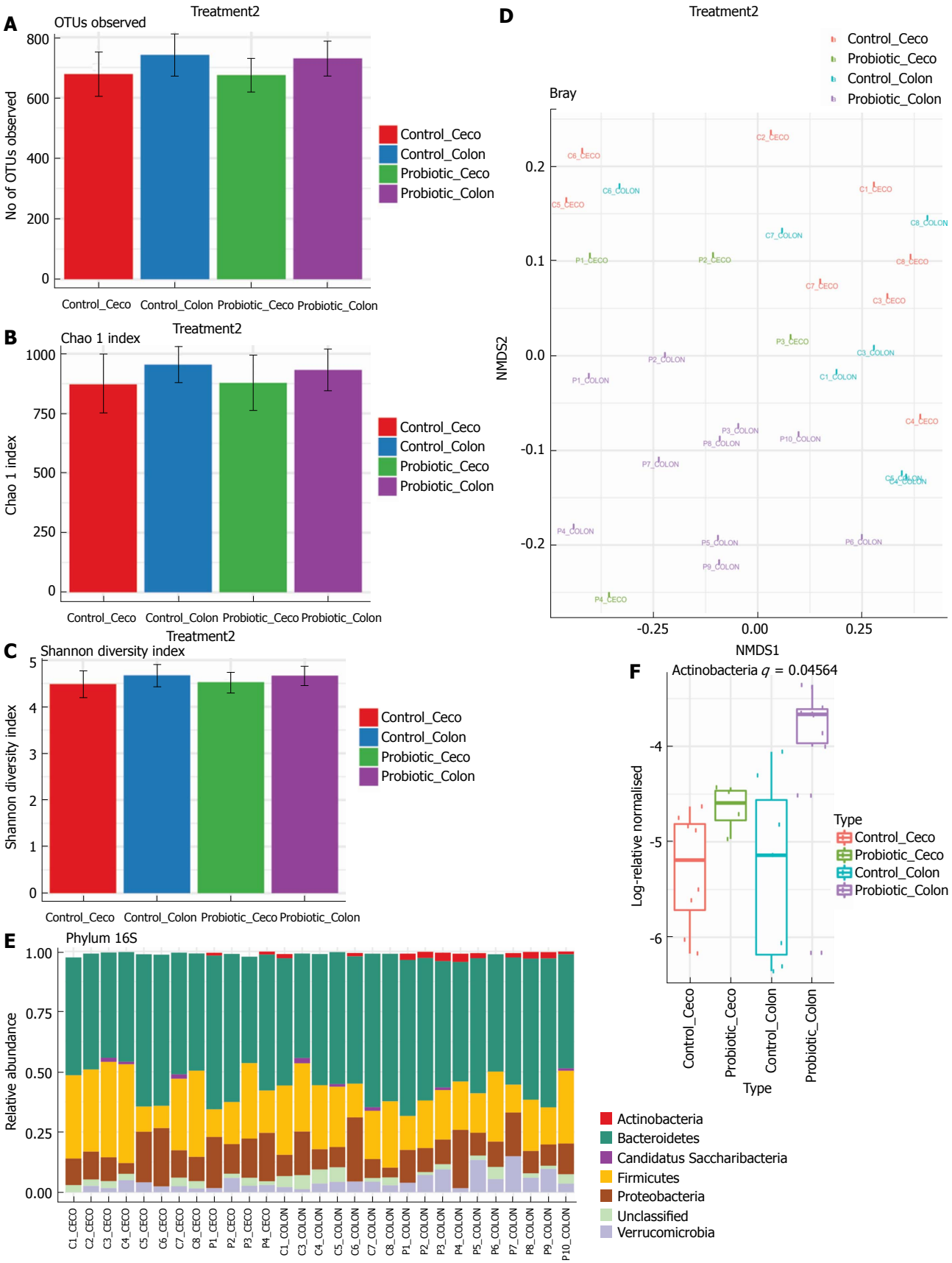

Figure 2 Probiotic supplementation changes the intestinal microbiota composition. A-C: Microbiota alpha-diversity in the faecal content of the cecum and colon from control and probiotic groups. Differences and comparisons of species richness (A), total number of operational taxonomic units, (B) Chao1 Richness Index, (C) Shannon Diversity Index. Data are expressed as means ± SEM. Control cecum, *n* = 8; Probiotic cecum, *n* = 4; Control colon, *n* = 7; Probiotic colon, *n* = 10, Kruskal-Wallis test. D: Non-metric multidimensional scaling (nMDS) analysis of colon and cecum faecal content from control and probiotic-treated animals. Each point represents a sample, groups identified by coloration, according to legend. E, F. Relative abundance at the phylum level in the cecum and colon from control and treated with probiotic Groups. E: Each bar represents an individual animal, the colour of each cell indicates the relative abundance of bacterial phyla. Taxa with relative abundance greater than 1% are shown. F: Box-plot represents log relative normalized abundance at the phylum level in the cecum and colon from control and probiotic-treated groups. 'C' for Control and 'P' for Probiotic. Only the significant *P* values obtained by the Kruskal-Wallis test are shown as *q* value.

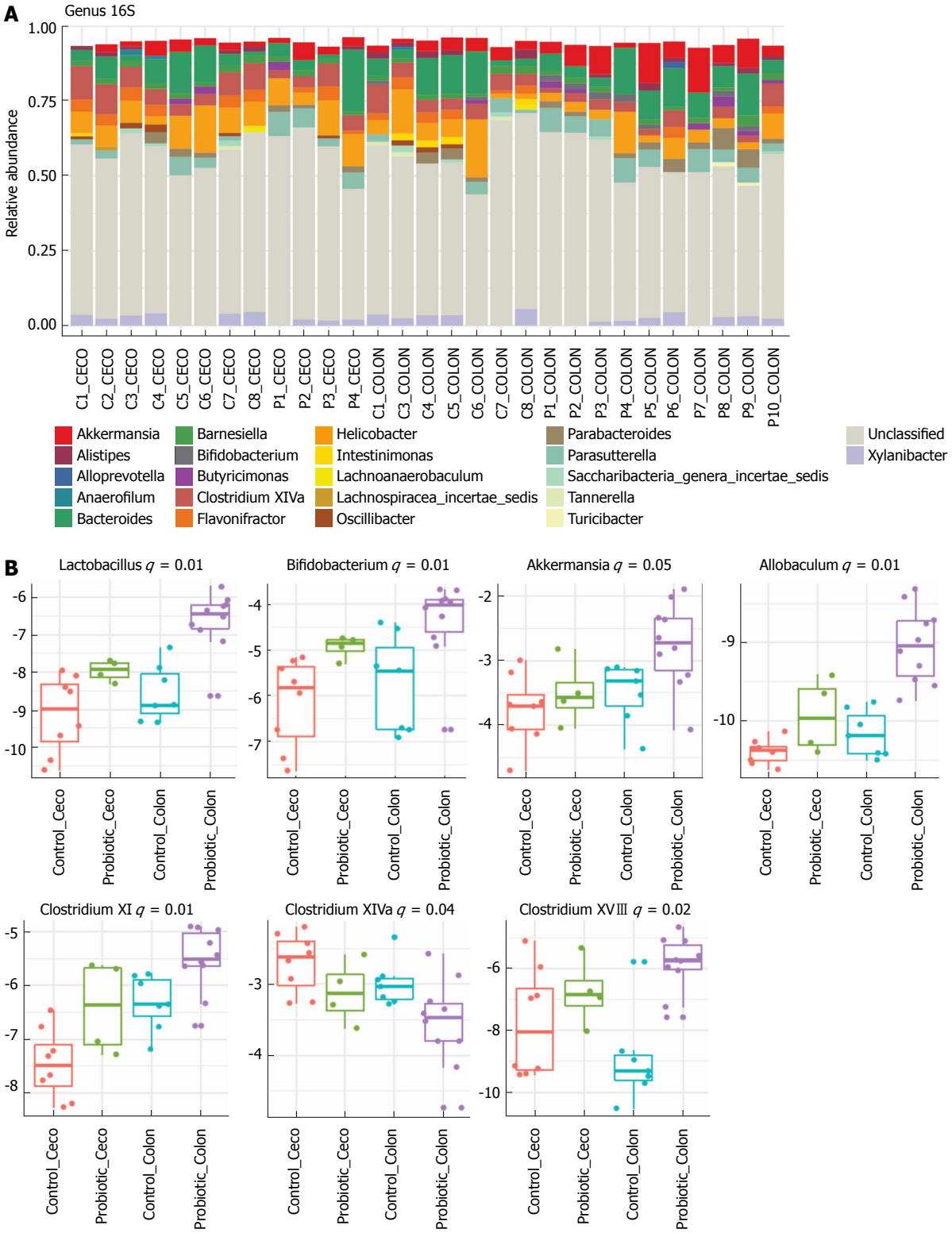

Figure 3 Probiotic supplementation changes the gut microbiota in the colon at the genus level. Relative abundance at the genus level in the cecum and colon from control and probiotic-treated groups at day 60 after colon cancer induction. A: Each bar represents an individual animal, and the colour of each cell indicates the relative abundance of the bacterial genus. Taxa with relative abundance greater than 1% are shown. 'C' for Control and 'P' for Probiotic. B: Box-plot represents log relative normalized abundance at the genus level in the cecum and colon from control and probiotic-treated groups. Only significant *P* values (*q* value) obtained by the Kruskal-Wallis test are shown. Control cecum, *n* = 8; Probiotic cecum, *n* = 4; Control Colon, *n* = 7; Probiotic colon, *n* = 10. Kruskal-Wallis test.

0.38] (Figure 4C).

In addition, serum cytokines were measured to assess whether probiotic supplementation could modulate systemic inflammation. There was an increase in important chemokines in the control group compared with the probiotic group: RANTES [control = 26.0 (± 8.4), *P* = 14; probiotic = 18.5 (± 6.9), *n* = 15; *P* = 0.03] and Eotaxin [control = 3010 (± 704.4), *n* = 14, probiotic

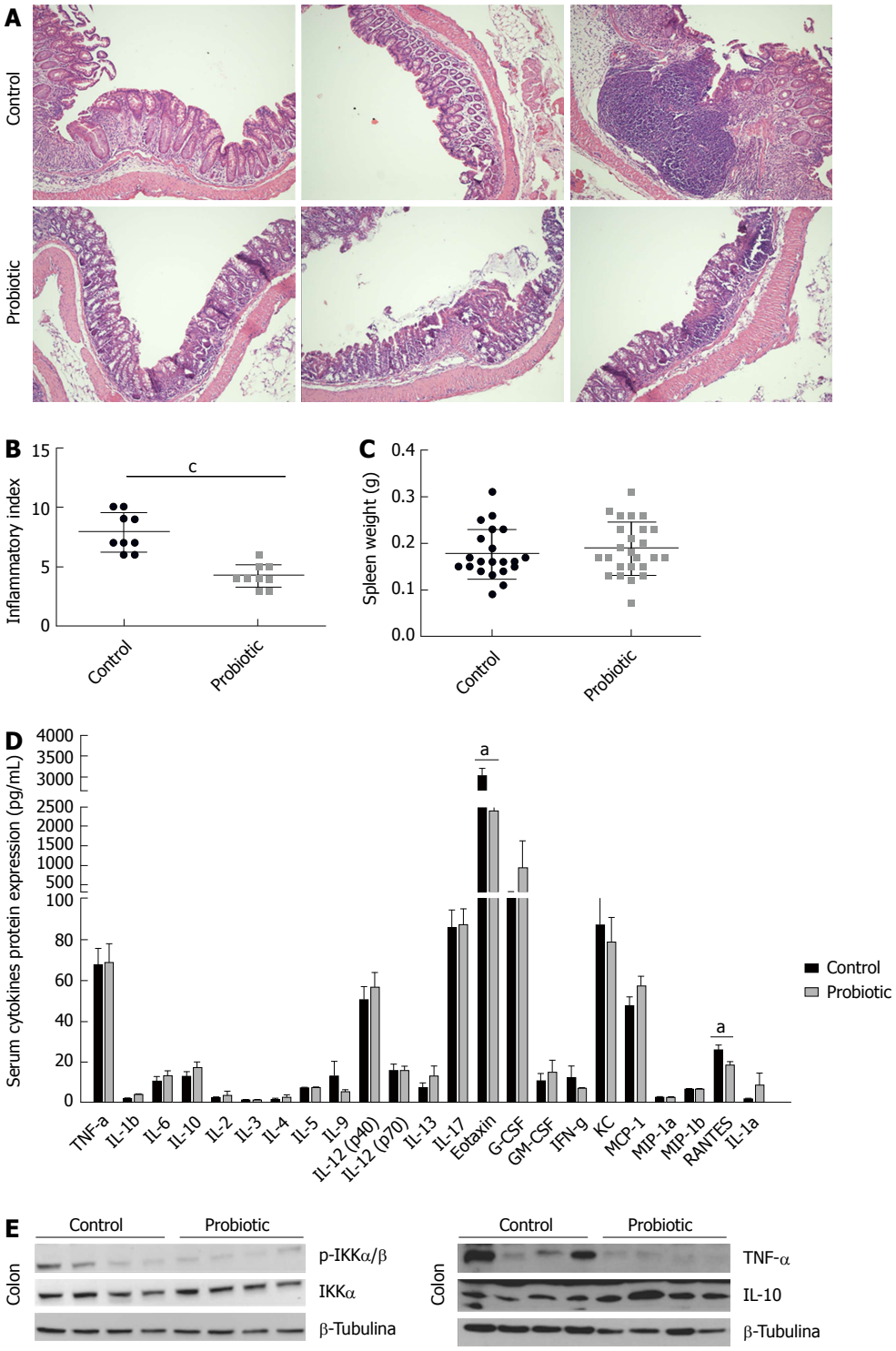

Figure 4 Probiotic supplementation modulates inflammation in colorectal carcinogenesis associated with colitis. Representative images (A) and inflammatory index (B) of mouse colons from control and probiotic groups at day 60 after azoxymethane injection stained with haematoxylin and eosin. $^{c}P < 0.001$ Mann-Whitney *U* test, data representative of two independent experiments presented as mean and standard deviation. C: Spleen weight in control group ($n = 21$) and probiotic ($n = 25$) mice. Mann-Whitney *U* test. Data from two independent experiments are presented as mean and standard deviation. D: Concentration of serum cytokines analysed by Bio-Plex Multiplex cytokine assay at day 60 after injection with azoxymethane. $^{a}P < 0.05$; Mann-Whitney *U* test. Data from two independent experiments, presented as mean and standard deviation. E: Representative western blot images of two independent experiments showing colon lysates of control and probiotic group mice; p-IKKα/β, IKKα, TNF-α, IL10 and β-tubulin.

= 2384 (± 854.4), $n = 15$; $P = 0.02$]. Increases in the mean levels of the cytokines IL-10 and IL-13 and a decrease in IFN-γ were observed, but these differences were not statistically significant. Other cytokines assessed did not differ between the control and probiotic groups (Figure 4D).

We further investigated whether intracellular inflammatory pathways in colonic tissue could be modulated

by probiotic supplementation. Our results demonstrated that the probiotic group had lower expression of the phosphorylated protein IKK, reduced TNF-α expression and increased IL-10 expression, which indicates less activation of inflammatory pathways (Figure 4E).

Probiotic supplementation does not modulate cytokine expression in the tumour microenvironment

In order to evaluate the expression of cytokines in the tumour environment, we evaluated the expression of cytokines in the tumour tissue by RT-PCR (Figure 5A-G) and by Bio-Plex Multiplex cytokine assay (Figure 5H) at day 60 after injection with azoxymethane. We observed an interesting but non-significant increase in the mRNA expression of the cytokine TGF-β, which was approximately 25% higher in the probiotic group [control = 1.00 (± 0.29) n = 17, probiotic = 1.24 (± 0.47) n = 21, P = 0.06] (Figure 5E). Changes in other cytokines were not statistically significant.

DISCUSSION

The present study demonstrates that supplementation with *Lactobacillus rhamnosus, Lactobacilus acidophilus* and *Bifidobacterium bifidum* reduced the colorectal tumour burden in mice, preventing colitis with a change in microbiota composition, reduction of inflammatory pathways in the colon, and modulation of cytokine and chemokine expression. To the best of our knowledge, no other study has evaluated the impact of the association of these strains in probiotic supplementation on the richness, diversity and abundance of the colon microbiota in colitis-associated cancer (CAC).

Prior reports showed that the isolated treatment with *Lactobacillus acidophilus, Lactobacillus rhamnosus or Bifidobacterium bifidum* are associated with tumour suppressive effects in colon cancer cell lines and in experimental tumour models[27-30]. Moreover, clinical studies showed that Lactobacillus and Bifidobacterium are frequently reduced in patients with intestinal bowel disease or CRC[31]. The enrichment or depletion of different microbial strains and the change in microbial diversity is considered essential for the promotion of inflammation, proliferation and neoplastic progression[32]. Here, we used the association of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* to assess if it can favourably alter the microbiota composition[33].

In this study, the alpha diversity (*i.e.*, the number of different taxa or microbial species that could be detected in one sample) was assessed by the Shannon index and richness by the Chao index in the gut microbiota of the colon, and there was no difference between the control and probiotic groups. Otherwise, a significant difference was observed in beta diversity (*i.e.*, the diversity in microbial community between different samples, accessed by the microbial composition abundances) and in the microbial composition at the genus and phylum levels. Based on these facts, it is possible to affirm that probiotic supplementation could change the structure of the microbiota.

The phylum Actinobacteria was increased in CAC supplemented with probiotics. Interestingly, Gao *et al*[34] using 16S rDNA sequencing observed that at the phylum level the number of Actinobacteria and Firmicutes decreased in the gut of CRC patients. Actinobacteria is a phylum of gram-positive bacteria, and the genus *Bifidobacterium* is one of its main components[35]; accordingly, probiotic supplementation increased the prevalence of this genus in CAC. Importantly, analysing the mucosa-adherent microbiota, Chen *et al*[36] identified reduced *Bifidobacterium*, *Faecalibacterium* and *Blautia* in CRC patients, while *Fusobacterium*, *Porphyromonas*, *Peptostreptococcus* and *Mogibacterium* were enriched. Similarly, colonic mucosa samples of patients with CRC presented a reduced amount of *B. longum* and *B. bifidum* compared with those in patients with diverticulitis[31]. These data suggest that probiotic supplementation alters the CAC microbiota to an anti-neoplastic one. Consistent with this hypothesis, probiotic supplementation increased the abundance of *Lactobacillus* in CAC. In particular, *Lactobacillus* not only prevents DMH induced colon carcinogenesis in rats[37,38] but also ameliorates inflammation in an experimental model of colon cancer[28,39,40]. *Akkermansia muciniphila* is another intestinal bacterium which may have potential anti-inflammatory properties in metabolic disorders, and it has been inversely associated with obesity, diabetes, cardiometabolic diseases and low-grade inflammation[41]. In other colitis model, such as interleukin-10 knockout mice, supplementation with *Lactobacillus plantarum LP-Onlly* ameliorates colon inflammation by microbiome alteration[42], while a combination of *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Enterococcus faecalis* improved epithelial-barrier function and reduced proinflammatory cytokines secretion[43]. *A. muciniphila* was reduced in ulcerative colitis patients[44], but was positively associated with CRC patients[45]. As a mucin-degrading commensal bacterium, it can impair intestinal barrier function, promoting colitis[46]. In contrast, other studies found that *A. muciniphila* increases the density of mucus-producing goblet cells, restoring the mucus layer[47]. In an experimental study, orally administered *A. muciniphila* extracellular vesicles protected against DSS-induced colitis, reducing proinflammatory cytokine expression, increasing colon length and reducing inflammatory cell infiltration of the colon wall[48]. We found an increase trend in *A. muciniphila* abundance in the probiotic group, which, in association with other microorganisms, may have prevented colitis in our study.

Furthermore, the abundance of short-chain fatty acid (SCFA)-producing bacteria, like *Allobaculum,* was increased in the probiotic group. SCFA (acetate, propionate, and butyrate) are produced through the fermentation of non-digestible carbohydrates

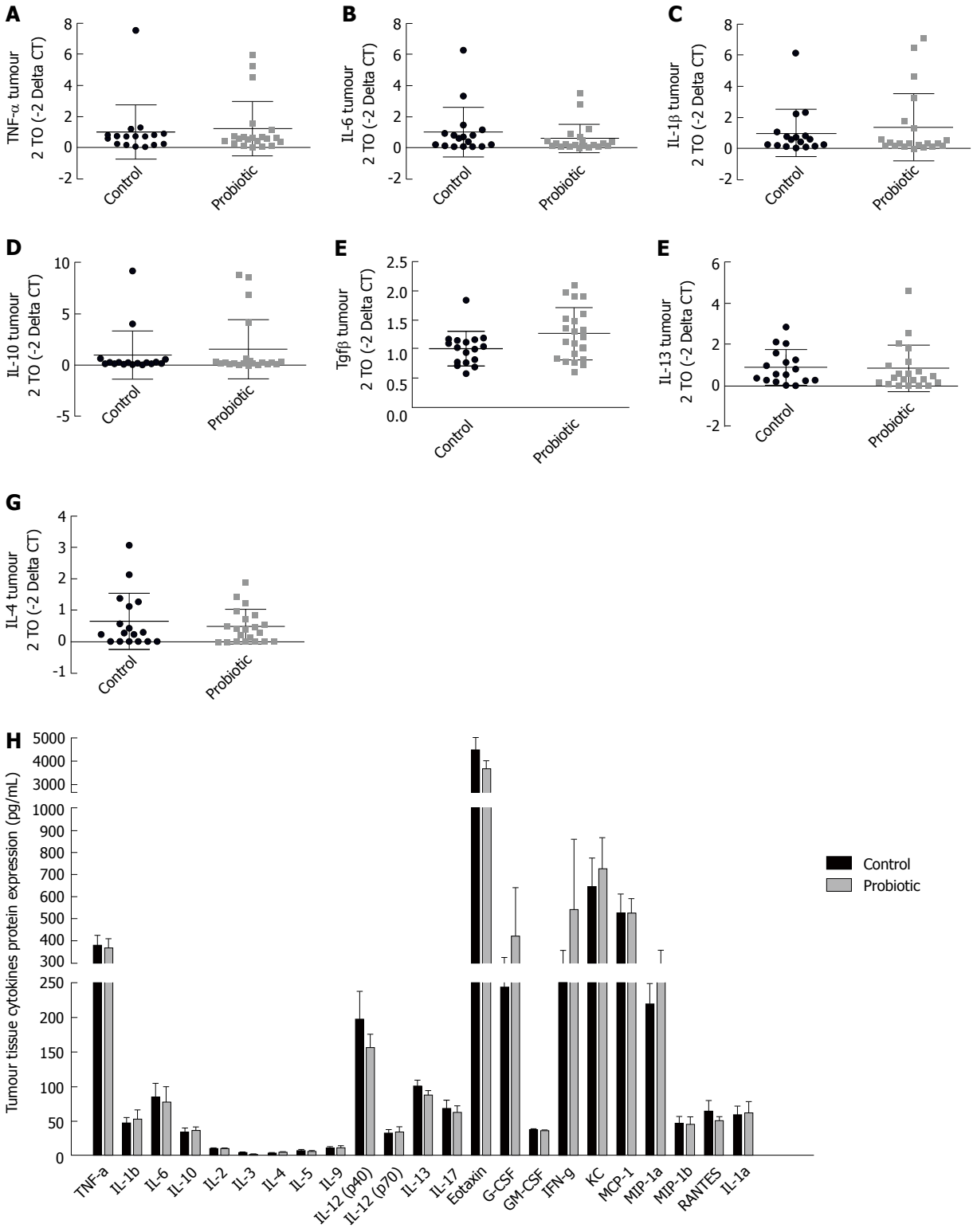

Figure 5 Probiotic supplementation does not modify the tumour microenvironment. Real-time RT-PCR analysis of TNF-α (A), IL-6 (B), IL-1β (C), IL-10 (D), TGF-β1 (E), and IL-13 (F), IL-4(G) in colon tumour tissue of control and probiotic groups; results are presented relative to those of *Gapdh*. H: Concentration of cytokines in the tumour tissue analysed by Bio-Plex Multiplex cytokine assay at day 60 after injection with azoxymethane. Mann-Whitney *U* test. Data from two independent experiments, presented as mean and standard deviation.

by intestinal bacteria and play an important role in maintaining intestinal health with anti-inflammatory and antineoplastic properties[49]. Antineoplastic properties of SCFAs are linked to the production of anti-inflammatory cytokines such as IL10 and TGF-β[50]. Likewise, *Bifidobacterium* and *S. thermophilus* also stimulate the release of TGF-β[51]. Considering that, we found an increase in the expression of TGF-β mRNA in tumour tissues of the probiotic group. It is possible that TGF-β may be involved in the anti-inflammatory and

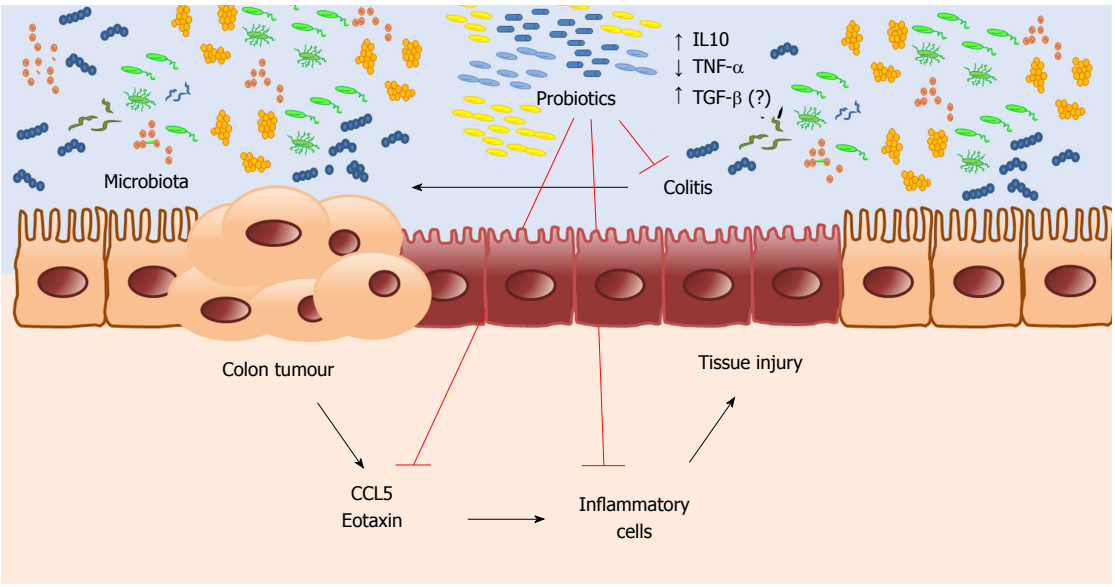

Figure 6 Microbiota modification by probiotic supplementation reduces colitis modulating cytokines expression preventing colon cancer development in mice.

anti-neoplastic effects of probiotic supplementation, and this deserves further investigation. Another mechanism by which probiotics can prevent inflammation is by modulating signalling pathways, inhibiting the PI3K/Akt and IKK/NF-κB pathway, thereby modulating cytokine and chemokine secretion[22,52]. In our study, probiotic supplementation reduced proinflammatory pathway activity, decreasing IKK activation in colon, suggesting a local effect. Notably, a similar result was observed in a study with ulcerative colitis patients patients where probiotic consumption increased IL-10, and decreased TNF-α and IL-1β, inhibiting NF-κB expression[53].

In accordance with a reduced activity of NF-κB, our data demonstrate that the probiotic-supplemented group presented reduced expression of CCL5/RANTES in serum. Given that CCL5/RANTES may promote tumour growth by stimulating proliferative pathways and angiogenesis and recruiting inflammatory cells[54-56], it is plausible to hypothesize that those chemokines are involved in tumour development, collaborating to reduced tumour burden in the probiotic-supplemented group. On the other hand, probiotic supplementation reduced the expression of eotaxin, a chemokine primarily responsible for eosinophil recruitment during inflammation, which may contribute to preventing the recruitment of inflammatory cells to the colon in the probiotic group. Eosinophils are potent proinflammatory cells, capable to produce and release cytotoxic proteins, cytokines, and metabolites reactive to oxygen, causing severe damage to the tissue. Eosinophils accumulation is common in patients with ulcerative colitis and active inflammatory bowel disease[57,58].

In aggregate these studies indicate that intestinal microbiota modulates carcinogenesis in different steps of carcinogenesis. Interestingly, the probiotic supplementation composition used in this study have its effects more pronounced in tumour initiation and promotion, as we found decreased tumour number and smaller tumour size in probiotic group.

It is plausible that probiotic supplementation can be included in clinical practice, preventing CRC in patients at higher risk of colitis. However, it is necessary to conduct a clinical trial to confirm this hypothesis. In conclusion, our results suggest a potential chemopreventive effect of probiotic supplementation on CRC. Microbiota changed by probiotic supplementation promote intestinal homeostasis and regulate the inflammatory response, reducing inflammatory cell infiltration by lowering chemokine expression, thus preventing CAC (Figure 6).

ARTICLE HIGHLIGHTS

Research background

Derangement in intestinal microbial composition impacts in mucosal inflammation, tumour promotion and neoplastic progression. Given that the intestinal microbiota can be modulated by several factors, and probiotic supplementation is an interesting alternative to re-establish intestinal eubiosis. Furthermore, in vitro studies and experiments with animal models demonstrated that several bacteria strains (probiotics) can modulate proliferative, apoptotic and inflammatory pathways; increase the innate immune response; produce anti-tumourigenic and anti-mutagenic compounds and destroy carcinogens; reduce genotoxicity; and increase intestinal barrier. Thus, probiotic modulation of intestinal microbiota has emerged as a potential chemopreventive agent.

Research motivation

Despite the idea that probiotic supplementation could prevent colorectal cancer (CRC), little is known about the supplementation of a mix of bacterial probiotic strains as well as its impact in the intestinal microbiota composition and neoplastic transformations of the intestinal mucosa. Our data can contribute to solve the gaps in the literature of whether this mix of probiotic, dose and time of supplementation used was able to alter the alpha and beta diversity of the intestinal microbiota, and how this treatment impact in colitis, serum cytokines and neoplastic development.

Research objectives

The aim of this work is to investigate the effect of supplementation of a *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*

mixture on the intestinal microbiota composition, inflammation and neoplastic alterations in the colon during the development of an experimental model of colitis associated colon cancer (CAC). Overall, this study intents to strengthen data from preclinical studies, encouraging clinical trials to investigate their role in preventing colitis and CRC in humans.

Research methods

We used an experimental model of CAC. C57BL/6 mice received intraperitoneal injection of Azoxymethane, followed by 3 cycles of 2.5% dextran sulphate sodium in drinking water, with an interval of 14 days between cycles. The intervention group received by gavage daily 0.6 billion CFU (colony forming units) each of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*, diluted in 200 μL of drinking water, while the control group received 200 μL of drinking water daily. Colon tissues were collected for inflammatory index analysis in histological sheets and western blotting to assess inflammatory proteins expression. Cytokines expression in serum and tumour tissue was performed by multiplex immunoassay, and in tumour samples were also used Real Time-PCR. Microbiota analysis was done from colon faeces using 16S rRNA sequencing method.

Research results

Probiotic supplementation reduces tumour incidence in a colitis associated colorectal model, we found decreased tumour number and smaller tumour size in probiotic group. In parallel, probiotic supplementation changes the gut microbiota in the colon. We did not detect any change in alpha diversity of the intestinal microbiota, but a difference in beta diversity and in the microbial composition at the genus and phylum level. In addition, probiotic supplementation reduced 46% the inflammatory index compared to the control group. Overall, these results highlight the potential for use of these probiotics mixture to human colitis to reduce inflammation and prevent colon cancer. Thus, further clinical trials are needed to confirm these preclinical insights.

Research conclusions

We found that supplementation with *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* during colitis associated colorectal carcinogenesis model changed intestinal microbiota, without altering richness and diversity of intestinal microbiota. *Lactobacillus* and *Bifidobacterium* increased in probiotic group and may be responsible for chemopreventive effect of probiotic supplementation on CRC. In summary, we suggest that probiotic supplementation could prevent CAC development by changes in microbiota composition which promotes intestinal homeostasis and regulates the inflammatory response, reducing inflammatory cell infiltration by lowering chemokine expression.

Research perspectives

The present study made biological plausible that probiotic supplementation can reduce inflammation and prevent CRC in patients with colitis. Therefore, clinical trials are needed to confirm this hypothesis and increase the therapeutic arsenal against this haunted disease.

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ORIGINAL ARTICLE

Basic Study

Ischemia/reperfusion injury in porcine intestine - Viability assessment

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Abstract

AIM

To investigate viability assessment of segmental small bowel ischemia/reperfusion in a porcine model.

METHODS

In 15 pigs, five or six 30-cm segments of jejunum were simultaneously made ischemic by clamping the mesenteric arteries and veins for 1 to 16 h. Reperfusion was initiated after different intervals of ischemia (1-8 h) and subsequently monitored for 5-15 h. The intestinal segments were regularly photographed and assessed visually and by palpation. Intraluminal lactate and glycerol concentrations were measured by microdialysis, and samples were collected for light microscopy and transmission electron microscopy. The histological changes were described and graded.

RESULTS

Using light microscopy, the jejunum was considered as viable until 6 h of ischemia, while with transmission electron microscopy the ischemic muscularis propria was considered viable until 5 h of ischemia. However, following $\geqslant$ 1 h of reperfusion, only segments that had been ischemic for $\leqslant$ 3 h appeared viable, suggesting a possible upper limit for viability in the porcine mesenteric occlusion model. Although intraluminal microdialysis allowed us to closely monitor the onset and duration of ischemia and the onset of reperfusion, we were unable to find sufficient level of association between tissue viability and metabolic markers to conclude that microdialysis is clinically relevant for viability assessment. Evaluation of color and motility appears to be poor indicators of intestinal viability.

CONCLUSION

Three hours of total ischemia of the small bowel followed by reperfusion appears to be the upper limit for viability in this porcine mesenteric ischemia model.

Key words: Viability; Histology; Reperfusion; Ischemia; Microdialysis; Jejunum; Porcine model

Core tip: Research on experimental methods to improve the surgeon's assessment of viability of ischemic bowel with higher accuracy than currently possible, requires an accurate reference model. We investigated viability assessment in a porcine model of warm ischemia on jejunum with mesenteric occlusion, followed by reperfusion. Our aim was to determine the time point of irreversible damage, to provide a reference model. We created parallel segmental models on the jejunum in 15 pigs and compared the results from visual inspection with histology and microdialysis. Three hours of ischemia followed by reperfusion appeared to be the upper limit for viability in this model.

Strand-Amundsen RJ, Reims HM, Reinholt FP, Ruud TE, Yang R, Høgetveit JO, Tønnessen TI. Ischemia/reperfusion injury in porcine intestine - Viability assessment. *World J Gastroenterol* 2018; 24(18): 2009-2023 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/2009.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.2009

INTRODUCTION

Evaluation of intestinal viability is essential in surgical decision-making in patients with acute intestinal ischemia[1-3], but can be challenging as the appearance of the ischemic or reperfused intestine can be deceptive[4]. The standard clinical method for intraoperative assessment of intestinal viability is evaluation of color, motility and bleeding of cut ends[3]. This method is not very specific and requires a high level of clinical experience[4,5].

There is a risk of short bowel syndrome if resection is performed too extensively, and on the other hand, a risk of peritonitis, sepsis and death if non-viable intestine is not removed[6]. The gold standard for determination of bowel viability is a second-look laparotomy (within 48 h) to reinspect areas of questionable viability[7]. Up to 57% of patients need further bowel resection at a later time, and this number includes patients undergoing second look surgery (40% of the patients)[8].

The intestinal wall consists of several tissue layers that have varying ability to tolerate ischemic insults. While the mucosa has a lower tolerance for ischemic damage than the muscularis propria, the mucosa has a very potent ability for rapid regeneration and repair[9]. When the muscularis propria and the muscularis mucosae are damaged, peristalsis and the movement of the villi will be lost. Regenerated scar tissue might not uphold sufficient peristalsis, and may lead to later stricture[2].

While intestinal ischemia may have a number of underlying causes, an early and essential element of the clinical treatment in nearly all cases is the restoration of perfusion[10]. However, it may cause both local and systemic responses, potentially creating damage far beyond the direct ischemic injury[11-13]. The extent of ischemia/reperfusion injury is variable and dependent on the underlying mechanisms, the duration of ischemia, the length of the affected segment and hypoxic tolerance of the tissue[10,14].

Experimental studies on intestinal viability have reported that the time before irreversible damage occurs varies between species, between anatomical locations (*e.g.* jejunum, ileum, or colon), and between the ischemia models used[15-17]. Rat intestine is reported

to be irreversibly damaged after 45 min of ischemia[18], whereas in juvenile pig jejunum irreversible damage to mucosal regeneration has been reported after 6.5 h of ischemia[19]. To judge the accuracy of clinical and experimental methods in the assessment of intestinal viability, histological analysis and/or patient outcome approaches have been used as the standard for comparison[4].

There is presently no standard classification method for the histological assessment of ischemia/reperfusion damage in the gut[20] and several approaches have been proposed, focusing on different aspects of the damage process[21]. Many previous studies of intestinal viability have concentrated on mucosal injury[13,22-25]. A commonly used histological classification system for ischemic mucosal lesions is based on the grading system proposed by Chiu *et al*[22], including modifications proposed by Park *et al*[26] to include evaluation of damage in the deeper layers of the intestine. Swerdlow *et al*[21] proposed a classification system, suggesting that mixing etiologic and morphologic terms should be avoided. This classification system has later been modified[27,28].

Microdialysis has been suggested as a way to monitor bowel ischemia[29], and can be used to measure changes in local metabolic substrate concentrations related to ischemia/reperfusion injury[30-32]. The principle is to place a tubular microdialysis membrane in the tissue of interest, to pump a slow and steady flow of isotonic fluid through the inside of the membrane and on to a sampling vial. The tubular semi-permeable membrane will allow low molecular weight substances in the area surrounding the probe to diffuse through the porous membrane due to differences in concentration gradient[33]. When using intraluminal microdialysis in the small intestine, the substrates of interest are primarily lactate and glycerol. The anaerobic metabolism in the ischemic cells leads to an increase in lactate, and glycerol is released as cell membranes deteriorate. Ischemia/reperfusion experiments have shown, however, that intraluminal microdialysis measurements of glucose and pyruvate can be unreliable[34,35].

In this study, we compared the results from visual inspection, intraluminal microdialysis and histology (light and transmission electron microscopy) with the aim of assessing the viability of porcine jejunum following segmental mesenteric occlusion with warm ischemia and further reperfusion. We evaluated the injury occurring in all layers of the intestinal wall. The overall aim was to determine when irreversible damage occurs, and to establish a reference for use with experimental approaches of viability assessment on the porcine jejunum.

MATERIALS AND METHODS

Animals and experimental design

The animal protocol was designed to minimize pain or discomfort to the animals and reduce the overall number of animals used. The experiment was approved by the Norwegian Food Safety Authority (FOTS ID 8304 and 12695) and conducted in accordance with Norwegian animal welfare guidelines (FOR-2015-06-18-761) and EU directive (2010/63/EU). We conducted the study on 15 Norwegian Landrace pigs, with a weight range 44.3-58.6 kg, 11 were females. Food was withheld 12 h prior to surgery. We used a segmental mesenteric occlusion (SMO) model utilizing several small bowel segments in the same pig[12,19,36,37], selecting 30 cm segments of the jejunum, starting 30 cm distal from the duodenum. More than 30 cm free intervals were maintained between the segments. Local ischemia was induced by atraumatic clamping of the arteries and veins of the jejunal mesentery on the selected segments[17,19], resulting in a 20-cm central zone of warm ischemia and two surrounding approximately 5 cm edge zones of marginal tissue hypoxia[38]. Reperfusion was initiated by releasing the clamps and verified by observing the return of color in the previously ischemic segments. We conducted a series of ischemia/reperfusion intervals (ischemia 1-16 h, reperfusion for 5-15 h post 1-8 h of ischemia, control 1-16 h) in order determine the occurrence of irreversible injury. At the end of the experiment, the animals were sacrificed by a lethal dose of potassium chloride (100 mmol).

Anesthesia and monitoring

Anesthesia was induced with intramuscular ketamine (Warner Lambert, Morris Plains, NJ, United States) 15 mg/kg, azaperone (Janssen-Cilag Pharma, Austria) 1 mg/kg, and atropine (Nycomed Pharma, Asker, Norway) 0.02 mg/kg. Tracheotomy was performed, and anesthesia was maintained with isoflurane (Abbott Scandinavia AB, Kista, Sweden) (1%-1.5%) and a mixture of air and O_2 to obtain an FIO_2 of 30%. Morphine (Alpharma, Oslo, Norway) 0.4-0.7 mg/kg/h was administered as a continuous intravenous infusion. Ventilation was adjusted to a pCO_2 of 5-6 kPa (37.5-45.0 mmHg). A continuous infusion of Ringer acetate 10-30 mL/kg/h was administered as fluid replacement.

Surgery

Surgery was performed under sterile conditions. Tracheostomy was performed initially for mechanical ventilation. The left internal jugular vein was cannulated with a triple lumen catheter for blood sampling, measuring of central venous pressure and infusion of fluids. Arterial pressure was measured through a catheter placed in a carotid artery, the urinary bladder temperature was measured with a thermistor probe. Arterial and venous blood gases were regularly measured throughout the experimental period. Pulse oximetry, heart rate, respiratory rate and expiratory pCO_2 were continuously monitored. The jejunum was made accessible through midline laparotomy. The mesentery of the selected jejunal segments were marked and clamped using Satinsky clamps[39].

Table 1 Comparison of modified Swerdlow *et al*[21,27,28] and Park/Chiu *et al*[22,26] systems for grading of histological damage on the intestine

Grade	Modified Swerdlow	Park/Chiu
0	No pathological change	Normal mucosa
1	Focal loss of surface epithelium	Subepithelial space at villus tips
2	Mucosal infarction (extensive loss of surface epithelium, loss of variable amounts of lamina propria, sparing of basal glands, intact muscularis mucosae)	Extension of subepithelial space with moderate lifting
3	Submucosal infarction (variable necrosis of submucosa, complete mucosal necrosis, intact muscularis mucosae)	Massive lifting down the sides of the villi, some denuded tips
4	Mural infarction (loss of muscularis mucosae, complete necrosis of mucosa and submucosa)	Denuded villi, dilated capillaries
5	Mural infarction (involvement of inner layer of muscularis propria, complete necrosis of mucosa and submucosa)	Disintegration of lamina propria
6	Transmural infarction (complete necrosis of the bowel wall)	Crypt layer injury
7		Transmucosal infarction
8		Transmural infarction

Peristalsis and color

The presence of peristalsis in the bowel segments was monitored by visual observation and palpation, and registered hourly for the duration of the experiments. We photographed the intestinal segments hourly to monitor alterations in color.

Microdialysis

CMA65 Custom made Microdialysis Catheter (65CMC) with 30 mm membrane length, 100 kDa cut-off (M Dialysis AB, Stockholm, Sweden) was perfused with 60 mg/mL Voluven (Fresenius Kabi Norge AS, Halden, Norway) for 30 min, before being inserted into the lumen of the selected jejunal segments, with a split-needle technique. The flow rate was adjusted to 1 μL/min using CMA 107 microdialysis pumps (CMA Microdialysis, Stockholm, Sweden). A baseline measurement was obtained (30 min) before the initiation of ischemia, and then for every hour during the experiment duration. An ISCUSflex Microdialysis Analyzer (M Dialysis AB, Stockholm, Sweden) was used to analyze the samples continuously after sampling, using Reagent set A (M Dialysis AB, Stockholm, Sweden). The reagent set was used to analyze glucose, lactate, pyruvate and glycerol. The ISCUSflex was set to normal linear range, 0.1-12 mmol/L (lactate) and 10-1500 μmol/L (glycerol). After a period of ischemia our results reached values above the linear range. Seven of the microdialysis catheters failed to operate normally and were excluded from the study.

Histology

We collected a total of 128 intestinal tissue samples from 5 pigs for light microscopy (LM) at selected time intervals from control jejunum, ischemic jejunum and reperfused jejunum. The biopsies were fixed overnight in buffered 10% formalin. The samples were then processed according to a routine protocol and embedded in paraffin wax, and 2-3 histological sections from each sample were stained with hematoxylin and eosin. The sections were reviewed with LM by two pathologists (HMR & FPR) and pathological changes in each layer of the intestine were assessed. The intestinal tissue damage was also classified using a system devised by Antonioli and Swerdlow, as modified by Hegde *et al*[27,28] and a modification of the grading system devised by Chiu[22], proposed by Park *et al*[26] (Table 1).

In addition, 58 samples at selected time intervals from 3 pigs were collected and fixed in a phosphate-buffered mixture of 2% glutaraldehyde and 0.5% paraformaldehyde overnight. From each sample, four specimens were subsequently embedded in an epoxy resin according to a standard protocol. Toluidine blue-stained semi-thin sections were used to select areas of interest and ultra-thin sectioning of one block. The ultra-thin sections were examined by transmission electron (TEM) microscopy by one pathologist (FPR). The focus was on cellular and subcellular changes as a basis of estimating tissue viability in the muscularis propria.

Statistical analysis

The microdialysis data was analyzed for distribution, skewness, kurtosis and homogeneity of variance to assess distribution. Continuous data were described with mean and SD and categorical data with counts and proportions. Comparisons of the intraluminal lactate and glycerol levels between the control and ischemia/reperfusion segments of jejunum were made using two-way repeated measures analysis of variance (RM ANOVA). For the ANOVA's the responses of interest were lactate and glycerol level, and the factors used were "case" (control, ischemic, reperfusion) and time duration [h]. *P*-values were adjusted for multiple comparisons using Holm-Sidak's correction. The ANOVA's were run using GraphPad Prism version 7.00 (GraphPad Software, United States).

RESULTS

All the animals (n = 15) were hemodynamically stable during the experiments. 10-20 min after reperfusion was initiated in a segment of the jejunum after a period of ischemia, there was an increase in heart rate (+20 to 60 beats per minute) that lasted for 5 to 30 min (increasing with the late reperfusion intervals), and there was also an initial decrease in mean arterial

Table 2 Clinical parameters during ischemia/reperfusion in porcine jejunum

Ischemia (h)	Observations on the ischemic jejunum	Minutes after reperfusion before color has returned (mean ± SD)	Observable peristalsis in No. of pigs	Reperfusion (h)	Observations on the reperfused jejunum	No. of pigs
0	Normal color					15
1	Purple	0.9 ± 0.1	15 of 15	8	Edema	15
2	Darker purple	2 ± 0.1	2 of 2	8	Edema, slight fibrinous coating	2
3	Darker purple	4 ± 0.3	13 of 13	8	Edema, fluid droplets, slight fibrinous coating	13
4	Darker purple	6 ± 0.7	4 of 4	8	Edema, fluid droplets, fibrinous coating, darker internal hue	4
5	Darker purple	15 ± 1.6	11 of 11	8	Edema, fluid droplets, fibrinous coating, darker internal hue	11
6	Darker purple	26 ± 3.3	3 of 4	8	Edema, fluid droplets, fibrinous coating, deeper red color, darker internal hue	4
8	Black	49 ± 9[1]	0 of 4	8	Edema, fluid droplets, fibrinous coating, deeper red color, darker internal hue	4
12	Patches of paler color					4
16	Necrotic					4

[1]There was a lot of internal bleeding in the jejunum, so determination of the time before return of color was difficult. Images in Figure 1.

blood pressure (5-25 Torr) lasting for 5-15 min, before returning to normal after increased fluid administration. SpO_2 (measured at the pig tail) was above 98% in all animals during the entire experiment. Mean body temperature increased from 38.5 ℃ at the start of the experiments to 40.5 ℃ by the end of the experiment.

Peristalsis and color

After initiating ischemia of a bowel segment, we observed a period of hyperperistalsis that lasted for approximately 30-40 min. Ischemia leads to a change in color of the involved tissue (Figure 1 and Table 2), and edema is the hallmark of reperfusion. Upon reperfusion, peristalsis was visible in all jejunal segments that had been ischemic for ⩽ 5 h and most of the segments that had been ischemic for 6 h. We observed an initial hyperemia, and a return of color even in the jejunum that had been ischemic for 8 h. In the samples that had been ischemic for ⩾ 2 h there was a gradual formation of a fibrinous exudate on the serosa after reperfusion. Following reperfusion, we observed the formation of small fluid droplets on the surface of the samples that had been ischemic for ⩾ 3 h, which was associated with a gradual increase in peritoneal fluid. We observed a darker "internal hue" in the samples that were reperfused after ⩾ 4 h of ischemia.

Microdialysis

Levels of the intraluminal lactate increased significantly during the first hour of ischemia ($P < 0.001$) from mean (SD) 0.65 (0.28) to 8.54 (3.43) mmol/L, peaking around 4-5 h of ischemia compared to the control (Figure 2). Following reperfusion after 1 h of ischemia, the intraluminal lactate level showed little change during the first hour of reperfusion with 10.42 (1.97) mmol/L compared to 13.69 (2.33) mmol/L in the ischemic tissue. In the second hour of reperfusion the lactate levels decreased significantly to 4.64 (1.36) mmol/L compared to 15.43 (2.47) mmol/L in the ischemic tissue ($P < 0.001$). In the series with ischemia duration > 1 h, the lactate levels decreased over the first hour following reperfusion. In the tissue that was ischemic for the whole duration of the experiment there was a gradual decrease in lactate level from mean (SD) 17.22 (3.48) mmol/L at 6 h of ischemic duration to 12.96 (2.01) mmol/L by the end of the experiment. Only in the jejunum that was reperfused after 1 hour of ischemia, did the lactate values approach pre-ischemic levels during the experiment. There was no significant change in arterial lactate throughout the experiment (data not shown).

The intraluminal glycerol level increased significantly from mean (SD) 5.7 (2.0) to 554.1 (215) μmmol/L during the first hour after the initiation of ischemia ($P < 0.001$), peaking around 6-8 h of ischemia compared to the control (Figure 2). In the segments that were reperfused after 1-3 h of ischemia, the glycerol levels continued to increase during the first hour of reperfusion, while decreasing during the first hour of reperfusion following longer ischemia intervals. The glycerol levels in the lumen of the reperfused intestinal segments approached the control level after 6 to 7 h of reperfusion, regardless of previous ischemic exposure. In the tissue that was ischemic for the duration of the experiment, there was a gradual decrease in glycerol level from mean (SD) 3180.4 (382.8) μmmol/L at 7 h of ischemia to 2780.2 (471.0) μmmol/L by the end of the experiment.

Histopathology

Light microscopy: LM of cross-sections of jejunum showed gradually increasing signs of injury in the ischemic tissue with time, and more pronounced injury following reperfusion. There was some variation in the pattern and extent of pathological changes between different samples from the same time point, and between different areas within the same samples, but the lesions were reproducible and the predominant

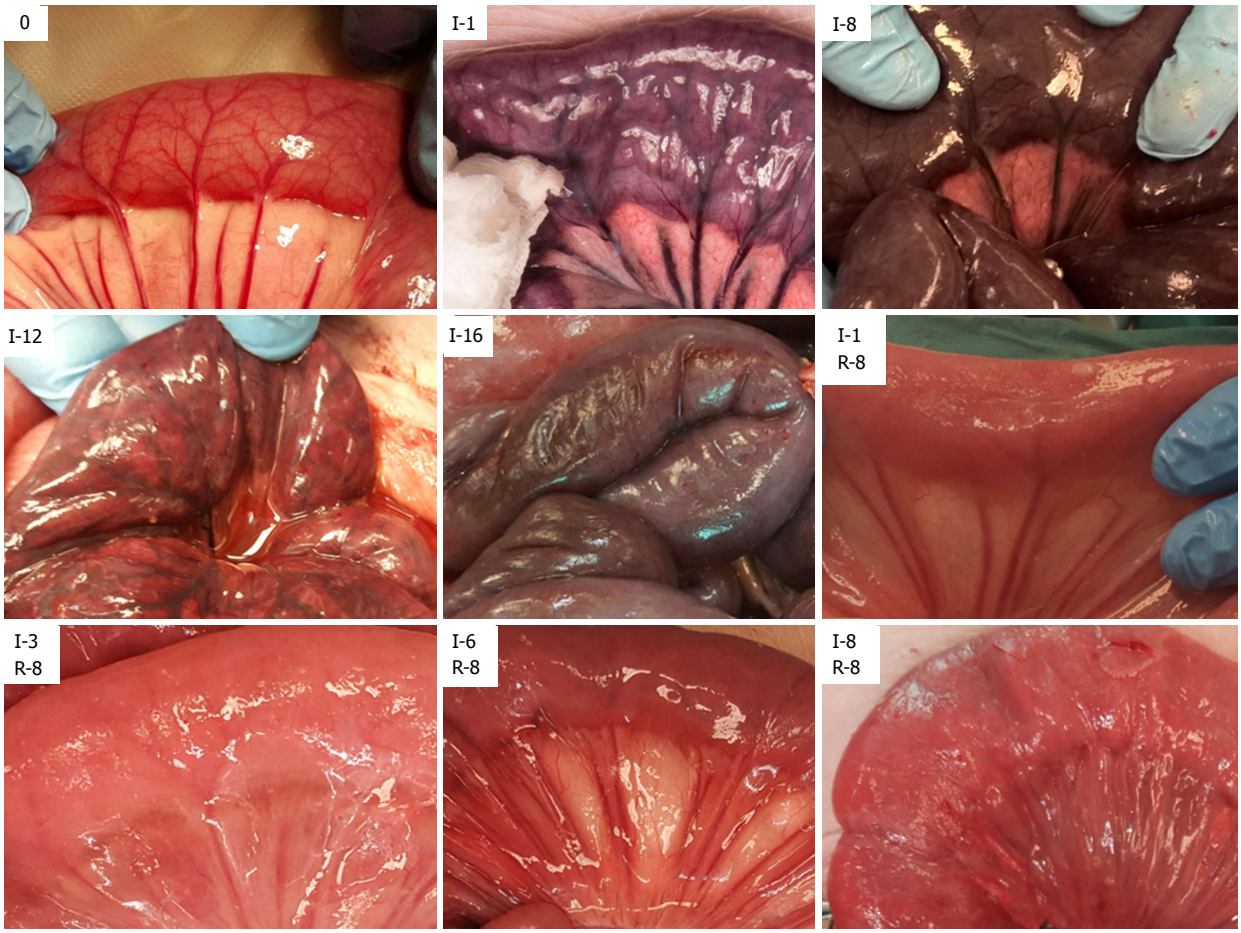

Figure 1 Jejunum at selected intervals of ischemia and reperfusion. 0: Perfused jejunum at the start of the experiment. I-1: 1 h of ischemia. I-8: 8 h of ischemia. I-12: 12 h of ischemia. I-16: 16 h of ischemia. I-1 R-8: 1 h of ischemia and 8 h of reperfusion. I-3 R-8: 3 h of ischemia and 8 h of reperfusion. I-6 R-8: 6 h of ischemia and 8 h of reperfusion. I-8 R-8: 8 h ischemia and 8 h of reperfusion. See Table 1 for description.

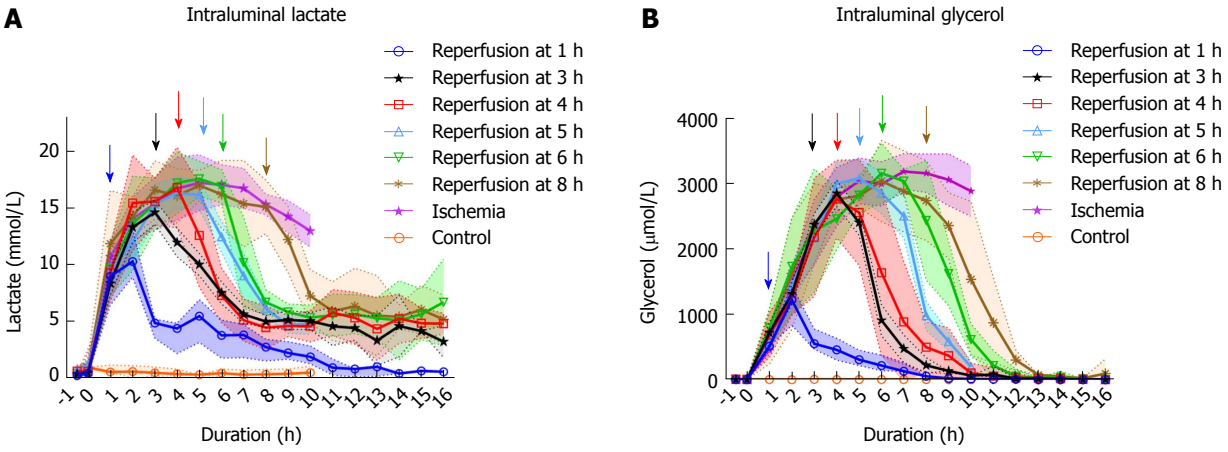

Figure 2 Intraluminal microdialysis in pig jejunum. A: Plots show intraluminal lactate median with 95%CI bands of the median. B: Plots show intraluminal glycerol median with 95%CI bands of the median. Both: Measurements starts with a baseline 30 min before the initiation of ischemia at t = 0. Colored arrows show time points for start of reperfusion. Ischemia and reperfusion at 1, 3 and 5 h n = 14. Reperfusion at 4, 6 and 8 h n = 4. Control n = 5.

findings at each time point are shown in Table 3. Based on the observations of total loss of crypt epithelium and pronounced smooth muscle cell shrinkage in the muscle layers, the samples from tissue exposed only to ischemia were considered irreversibly damaged by ischemia at 6 h exposure.

After one hour of ischemia and 8 h of reperfusion, there was increased apoptosis in the crypt epithelium, mild inflammation with neutrophils mainly in capillaries in all layers of the intestine, edema in the subserosa and submucosa and signs of focal injury to the outer layer of the muscularis propria. After 3 h of ischemia

Table 3 Summary of main findings from light microscopy of 128 biopsies from 5 pigs at selected intervals of ischemia/reperfusion time

Ischemia	1 h isc, 8 h rep	2 h isc, 8 h rep	3 h isc, 8 h rep	4 h isc, 8 h rep	6 h isc, 8 h rep	8 h isc, 8 h rep	Control
I-1: Early loss of SE[1]	I-1: Early loss of SE[1]	I-2: Total loss of SE[2]	I-3: Early loss of CE, congestion and bleeding LP[2]	I-4: Total loss of SE, focal damage to outer layer of MP[2]	I-6: Total loss of CE, damage to LP, MM. Bacteria in LP[2]	I-8: Damage to all components[3]	N-0: Normal[1]
I-2: Total loss of SE[2]	I-1/R-1: Total loss of SE, apoptosis in CE, light N[2]	I-2/R-1: Apoptosis in CE, light N, congestion and focal bleeding in LP[2]	I-3/R-1: Apoptosis in CE, N, wavy myocytes in MP[2]	I-4/R-1: Focal damage to both layers of MP (most to outer layer)[2]	I-6/R-1: Damage to all components[3]	I-8/R-1: Damage to all components[3]	N-6: Few instances of apoptosis in CE, light N and light edema in MP[1]
I-3: Early loss of CE[2]	I-1/R-3: Focal damage to outer layer of MP[2]	I-2/R-3: Early regeneration of SE, congestion, bleeding and necrosis in LP, apoptosis in CE, interstitial inflammation in MP[2]	I-3/R-3: Edema, inflammation, and focal necrosis in outer layer of MP[2]	I-4/R-3: Total loss of CE, NGR, cell disintegration in MM and MP[3]	I-6/R-3: Damage to all components[3]	I-8/R-3: Damage to all components[3]	N-12: Few instances of apoptosis in CE, light N and light edema in MP[1]
I-4: Focal damage to outer layer of MP[2]	I-1/R-6: SE regenerated. Focal damage to outer layer of MP[1]	I-2/R-6: Regeneration of SE, wavy myocytes and focal necrosis in MP[2]	I-3/R-6: Most of CE is lost, wavy myocytes and focal necrosis in MP[2]	I-4/R-6: Total loss of CE, NGR, loss of myocytes, disintegration[3]	I-6/R-6: Damage to all components[3]	I-8/R-6: Damage to all components[3]	
I-5: Damage to inner layer of MP[2]	I-1/R-8: SE regenerated. Focal damage to outer layer of MP[1]	I-2/R-8: Regeneration of SE with focal loss and erosion, focal damage to the MP with wavy myocytes and necrosis[2]	I-3/R-8: Most of CE is lost, wavy myocytes and focal necrosis in both layers of MP[2]	I-4/R-8: Damaged SE, CE, MM, submucosa, MP, PM[3]	I-6/R-8: Damage to all components[3]	I-8/R-8: Damage to all components[3]	
I-6: Total loss of CE, damage to LP, MM and bacteria in LP[3]							
I-7: Hemorrhage in subserosa, peritonitis, and damage to all components[3]							
I ⩾ 8: Damage to all components[3]							

Each column shows the results from a series of tissue samples, with time progression from the top to the bottom. The table is indexed using "I" for ischemia, "R" for reperfusion and "N" for normal perfusion, followed by a number showing the hour duration. The results are indexed by a superscript number by the end of each sentence. [1]Normal/light changes, [2]Visible cell damage, but still probably viable, [3]Probably irreversible cell damage. CE: Crypt epithelium; LP: Lamina propria; MM: Muscularis mucosae; MP: Muscularis propria; N: Neutrophils; SE: Surface epithelium.

and 8 h of reperfusion there was focal damage to all layers. After 4 h of ischemia and 8 h of reperfusion there was a total loss of crypt epithelium, extensive shrinkage and loss of myocytes in the outer layer of the muscularis propria, suggesting likely irreversible damage (Figure 3). In intestine subjected to 6-8 h of ischemia followed by 1 h of reperfusion, there were signs of damage to all components of the intestinal wall.

Histological damage according to grading systems: The predominant findings at each time point are shown in Figure 4. The highest score in both grading systems was reached after 8 h of ischemia. The reperfused tissue received a full score for intervals ⩾ 4 h of ischemia followed by 2 h of reperfusion. The observed sequence of ischemia/reperfusion injury did not necessarily follow the outwards direction from the mucosa to the outer muscular layer, as the grading systems suggest (compare Tables 3 and 4 with Table 1 and Figure 4).

Table 4 Summary of main findings from transmission electron microscopy of porcine jejunum at selected intervals of mesenteric occlusive ischemia and reperfusion

Ischemia (h)	Observations	Ischemia/reperfusion (h/h)	Observations
0	Intact musculature. Some variation in the electron density in the muscle cells, focal swollen mitochondria's with vacuolized matrixes[1]		
1	Intact musculature. Discrete intercellular edema. Lymphocytes in the interstitial space. Increased variation in the electron density in the muscle cells. Some cells have increased electron density (darker). Some of the mitochondria are more prominent. Some minimal fat vacuoles are visible[2]	1-3	Inflammation, cell death, sparse fine-vacuolization of the sarcoplasm, slightly swollen mitochondria[2]
2	More prominent variation in electron density between muscle cells. Increased number of vacuoles, some of them are fat vacuoles. Focal edema, thickening of the mitochondrial cristae. Some lysosomes with membrane fragments[2]	2-3	Inflammation, cell death, more comprehensive fine-vacuolization of the sarcoplasm, slightly swollen mitochondria[2]
3	Same results as at 2 h, but a few more interstitial immune response cells are visible. Monocytes, macrophages, and a few granulocytes. Vacuoles in the sarcoplasm. Slightly swollen mitochondria[2]	3-3	Inflammation, cell death, more comprehensive fine-vacuolization of the sarcoplasm, slightly swollen mitochondria, focal single cell necrosis, swollen cell nuclei[2]
4	Same changes as at 3 h, but the changes are more prominent as the cells with higher electron density are more condensed, and there are more vacuoles around the mitochondria[2]	4-3	Pronounced cell shrinking/cell death, swollen cell nuclei, loss of cohesion, interstitial edema[3]
5	Focal edema, variations in electron density, thickening of the mitochondrial cristae, vacuoles in the sarcoplasm, swollen mitochondria, interstitial lymphocytes/monocytes/granulocytes, loss of plasma-membrane and coherence, focal single cell necrosis[3]	5-3	Increased cell shrinking/cell death, swollen cell nuclei, loss of cohesion, interstitial edema[3]
6	Necrosis, focal large vacuoles in some mitochondria[3]	6-3	Increased cell shrinking/cell death, swollen cell nuclei, loss of cohesion, interstitial edema[3]
7	Necrosis with macrophages. Non-necrotic cells appear like the cells at time intervals 3-6 h[3]		
8	Like the results at 7 h[3]		

Changes in the muscularis propria and serosa are described (3 pigs, a total of 58 samples). The results are indexed by a superscript number by the end of each sentence. [1]Normal/light changes, [2]Visible cell damage, but still probably viable, [3]Probably irreversible cell damage.

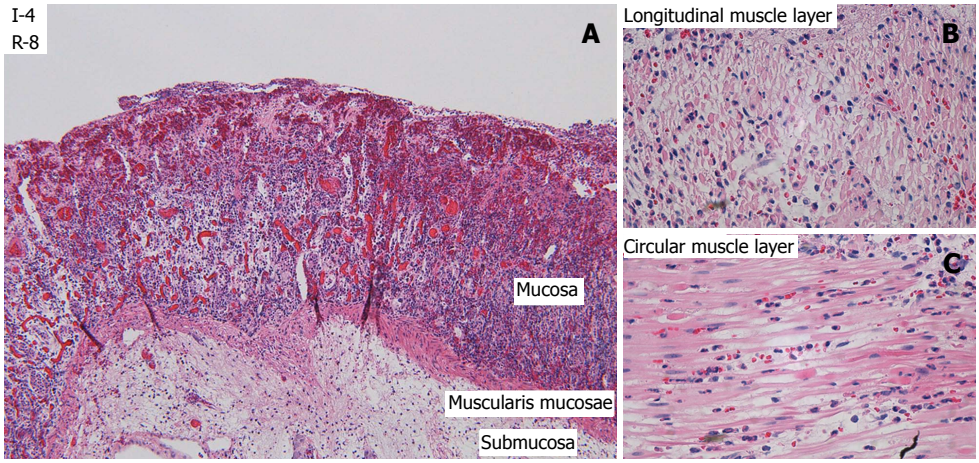

Figure 3 Light microscopy of selected structures of the jejunum after 4 h of ischemia and 8 h of reperfusion. A: Mucosa and submucosa (HE, × 10), showing necrotic villi, total loss of crypt epithelium, shrinkage of myocytes in the muscularis mucosae, and edema in the submucosa. B: Longitudinal (outer) layer of the muscularis propria, showing edema and extensive shrinkage and loss of myocytes (HE, × 60). C: Circular (inner) layer of the muscularis propria, showing edema and extensive myocyte damage (HE, × 60).

Transmission electron microscopy: Using TEM on the muscularis propria and serosa we observed a gradual increase in damage to the cell structures during ischemia (Table 4, left columns), with probably irreversible damage in the muscularis propria after 5 h of ischemia. Interestingly, even at 7 to 8 h of ischemia, focal areas of muscle cells still appeared viable, illustrating heterogeneity in the development of ischemic damage to the muscularis propria.

There was reperfusion induced inflammation and cell death of varying degrees in all the tissue that had been subjected to ischemia. After 3 h of ischemia and 3

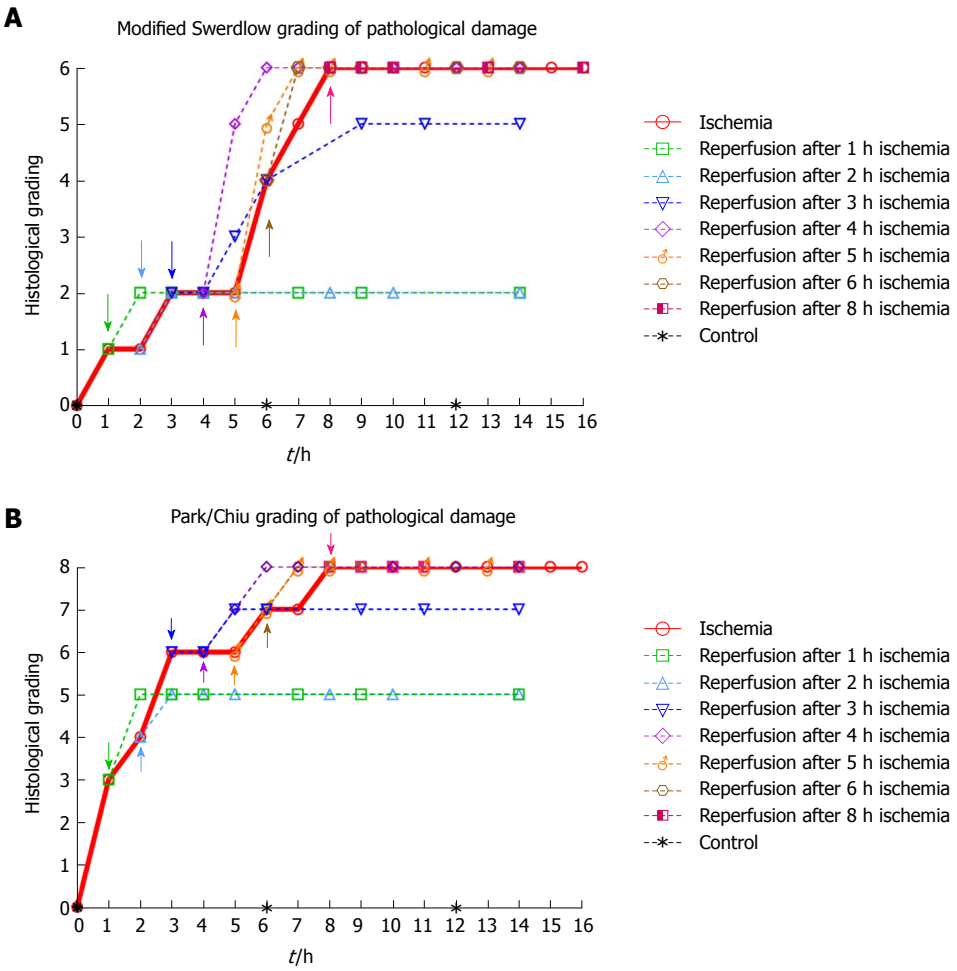

Figure 4 Histological grading of pathological damage (5 pigs, *n* = 128 biopsies total) at selected ischemia/reperfusion intervals. Colored arrows show time points for start of reperfusion. Stippled lines show progression of injury following reperfusion. A: Modified Swerdlow *et al*[21,27,28]. B: Park/Chiu *et al*[22,26].

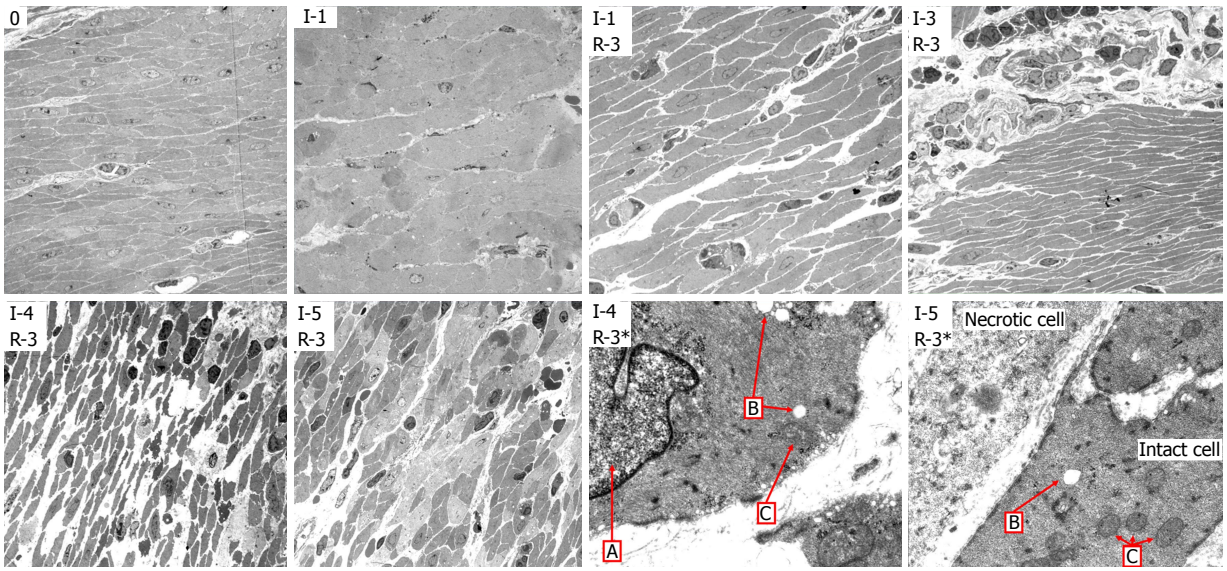

Figure 5 Transmission electron microscopy of jejunum (muscularis propria) sampled at selected time intervals of ischemia and reperfusion. Images are indexed with I = ischemia hours and R = reperfusion hours. 0: Intact muscle. I-1: Mild intercellular edema, with increased variation in the electron density in the muscle cells. Some minimal fat vacuoles are visible. I-1 R-3: Focal/single cell necrosis with inflammatory response, low grade fine-vacuolization of the sarcoplasm. I-3 R-3: Active interstitial inflammation, swollen muscle cell nuclei. I-4 R-3: Severe interstitial edema and loss of coherence among muscle cells. Swollen nuclei and focal, mostly single cell necrosis. I-5 R-3: Focal multi cell necrosis, interstitial inflammation, vacuolization of sarcoplasm. I-4 R-3*: Swollen nucleus (A), vacuolated sarcoplasm (B) and swollen mitochondria (C). I-5 R-3*: Necrotic muscle cell adjacent to a more intact cell with some vacuoles (B) and slightly swollen mitochondria (C).

h of reperfusion (Table 4, left), there was inflammation, cell death, slightly swollen mitochondria, and swollen cell nuclei, and the muscle tissue appeared to be approaching irreversible damage. After 4 h of ischemia and 3 h of reperfusion (Table 4, right), there was pronounced cell shrinking/death, swollen cell nuclei, loss of cohesion, substantial interstitial edema and the muscle tissue no longer appeared viable. Figure 5 shows TEM images with typical observations described in Table 4.

DISCUSSION

The viability of ischemic small bowel is determined in a clinical setting by observation of color, peristalsis and bleeding from cut ends. As this method is not very specific and requires a high level of clinical experience[5], there is a need for increased accuracy of the viability assessment[4]. Intraoperatively, decision on the resection margin is the most important factor contributing to postoperative mortality and morbidity[40,41]. We approached the question of viability assessment in ischemic and reperfused porcine jejunum by using microdialysis and by histological assessment of pathological changes. Microdialysis allowed monitoring of metabolic changes related to ischemia and reperfusion. Presumed irreversible tissue damage was detected after shorter duration of ischemia using TEM than with LM. Subsequent reperfusion aggravated ischemic damage to the jejunum. Likely irreversible damage (when including the effects of reperfusion) occurs between 3 and 4 h of full mesenteric warm ischemia in the porcine jejunum, indicating a time limit for viability in the model.

Visual inspection

While return of color and peristalsis does not correlate uniformly with intestinal viability[2,42], these are the most common criteria in the clinical assessment of intestinal viability[3]. A small variation in the nuance of darkness was the only change in color from 2-9 h of full occlusion ischemia, showing that intestinal color alone is a poor indicator of viability. The later change in appearance from dark (8 h), to patchy colored (11-12 h), to necrotic (15-16 h), indicates the time window between the initiation of full occlusion warm ischemia and the presence of pronounced necrotic bowel in the SMO model.

We observed return of color and peristalsis (Table 2) in intestine that histologically contained areas of probably irreversible damage (Table 3). Following reperfusion, the increase in time before return of color associated with an increase in ischemic exposure, indicating that the time before return of color is affected by the level of tissue injury. However, confounding effects such as internal bleeding and edema in the intestinal wall may have reduced the accuracy of the return of color assessment after the long reperfusion intervals.

Macroscopically, fibrin exudate was seen on the serosal surface (Table 2) on the segments that had been ischemic for more than 1 hour. In addition to being triggered by ischemia/reperfusion[43], the formation of fibrinous exudate on the serosa was probably exacerbated by handling and exposure of the intestine to foreign material during the course of the experiment[44].

Microdialysis

Using microdialysis to measure intraluminal lactate and glycerol, we were able to closely monitor the onset and duration of ischemia, and the onset of reperfusion (Figure 2). In the segments that were reperfused after $\geqslant$ 6 h of ischemia, we observed increasing leakage of fluid from the intestines into the abdominal cavity and increasing amounts of fluid accumulating inside the lumen. Granger *et al*[45] reported a doubling of vascular permeability during ischemia and a fourfold increase in vascular permeability after reperfusion. This probably dilutes the luminal lactate and glycerol concentrations, limiting the accuracy of intraluminal microdialysis during prolonged ischemia/reperfusion experiments[46]. The phenomenon is expressed by a gradual decrease in lactate and glycerol levels in the ischemic intestine past the 6-h duration.

Intraluminal lactate and glycerol levels have been reported to mirror the permeability (polyethylene glycol 4000) of the intestinal mucosa after ischemia, and lactate more precisely so than glycerol[47]. The lactate and glycerol levels started to decrease before reperfusion and dropped after reperfusion even in severely ischemic intestine (8 h), where we observed histological damage to all layers (Table 3). This suggests that the relationship between permeability and lactate/glycerol levels may be valid only after shorter periods of ischemia, and that our late results may be confounded by the dilution effect of leakage into the lumen.

In comparison to previous experiments using intraluminal microdialysis in ischemia/reperfusion of the small intestine in pigs[30,34,35,48,49], we have monitored the intestine over a longer period of ischemic time and over more ischemia/reperfusion intervals than previously reported. Interestingly, Solligard *et al*[47] monitored a single clamp for 9 h of reperfusion after 1 h of ischemia with similar results as ours.

After start of reperfusion, there appears to be no clear difference in the time course of metabolic marker concentration between reversibly and irreversibly damaged tissue, indicating that prediction of viability based on intraluminal microdialysis alone is unreliable. Ideally, placement of microdialysis catheters into the intestinal wall would be preferable, as this would circumvent the late ischemia/reperfusion effects related to intraluminal leakage and dilution. Still, intraluminal microdialysis has been recommended over microdialysis catheters inserted into the intestinal wall, because of the reported poor reliability of the latter method[30,35,47,49-53]. The present results confirm

that intraluminal microdialysis has high specificity and sensitivity for detecting and monitoring ischemia in the small intestine.

Histology and grading

LM (Figure 3, Table 3) and TEM (Figure 5, Table 4) showed a gradual increase in injury in the ischemic tissue with probable irreversible damage appearing around 6 h and 5 h, respectively, indicating that the pathological changes related to viability are visible somewhat earlier on the ultrastructural level than with LM. Tissue that still appeared viable after 4 h of ischemia was considered irreversibly injured after subsequent 3 h of reperfusion, indicating the limit of viability in the model.

When investigating what others have reported with respect to a viability limit in the porcine jejunum, we did not find much information. In most papers discussing viability in the small intestine, observations are reported as histological grading scores or as morphological observations[20], but few contain explicit statements about viability. The most common time duration reported for porcine intestine related to viability is that it takes approximately 8 h of full ischemia to induce transmural necrosis[22,54]. We observed the same result in the present study (Table 3, Figure 4).

Chan *et al*[18], reporting that irreversible damage in porcine jejunum, defined as lack of mucosal regeneration in samples taken 24 h after reperfusion, occurred after 6.5 h of ischemia followed by reperfusion[19]. We acknowledge that mucosal necrosis will heal completely in most cases, except in cases with necrosis of long mucosal segments with substantial damage to the crypt layer, where there is a risk of complications due to hemorrhage and fluid loss[15,21]. The mucosa can regenerate on injured segments of intestine that do not develop into transmural infarction. However, such segments may develop persistent injury with large degree of fibrosis and stricture formation[21]. The exacerbation of injury following reperfusion indicates that reperfusion is a major contributor to injury in the porcine SMO model.

As the Park/Chiu grading system was created to be sensitive to early mucosal changes, the initial grading after one hour of ischemia is 3, indicating that a finer resolution than 1 h of ischemia should be used to utilize its potential. The grading system may have been designed for assessment of inflammatory diseases and the status of cold preserved tissue for transplantation, rather than with respect to overall viability. The Swerdlow grading system has a more evenly distributed resolution with respect to injury in the whole intestinal wall, including two levels of injury with respect to mural infarction. Nevertheless, both systems arrive at similar results, as the structures are similar. We agree with Quaedackers *et al*[20] that a better description of the last grades of the Park/Chiu system would further strengthen its suitability.

We found that more than 3 h of ischemia gave a full score in both grading systems within two hours following reperfusion (Figure 4). This indicates that to assess jejunal viability using histology after an ischemic event of unknown duration, at least two hours of reperfusion is needed before the histological sampling will accurately illustrate the outcome. We generally observed slightly higher levels of injury than Blikslager *et al*[55] in a similar model used on the ileum in pigs, and Chan *et al*[19] in a similar model on the jejunum in two juvenile pigs. As the ileum is more resistant to ischemic damage than the jejunum we expected a slightly higher injury grade in the jejunum.

In the samples from the reperfused tissue that had been exposed to only one hour of ischemia, there was visible regeneration of the epithelial cells after 3 h of reperfusion, with a large degree of regeneration after 6 h. This is similar to what has been reported previously both in humans[56] and pigs[9,57].

An important observation from the present study is that the sequence of ischemia/reperfusion injury using the SMO model does not necessarily follow the outwards direction from the mucosa to the outer muscular layer, as most grading and classification systems suggest[16,20]. Rather, the ischemic damage may be patchy and somewhat unpredictable, as we observed tissue damage in the outer layer of the muscularis propria while the inner muscular layer still appeared viable. This is illustrated when comparing Figure 4 (histological grading) with Table 3 (morphologic observations).

Evaluation of tissue viability based on histological assessment is difficult[58], as the samples are small and lesions are heterogeneous in composition and distribution[59] with areas of viable and necrotic tissue in the same tissue sample. Predicting the healing potential of the various intestinal layers after ischemia/reperfusion is also challenging. Although we observed injury to the jejunal wall that we considered irreversible, the ability to regenerate is likely to vary with the total volume of damaged tissue, making exact assessments from tissue samples difficult. With respect to the observation of heterogeneous injury, Guan *et al*[60] speculated that this may be related to difference in the flow in the mesenteric versus antimesenteric side of the small intestine.

The model

We selected the pig model for viability assessment of the small intestine, as it has important anatomical and physiological similarities to humans[61], the pathophysiology of ischemia/reperfusion in the porcine model is similar to humans[12], and because the pig model has been suggested as a reference standard in intestinal transplantation research. The SMO model[17] was selected as it provides a well-defined area of ischemic injury affecting the whole intestinal wall in the occluded segment[12], as opposed to the commonly used intestinal ischemia model of occlusion of the superior

mesenteric artery[17,62]. The SMO model simulates ischemic injury as caused by strangulation-ileus.

A 50 kg pig has approximately 15 meters of small intestine[63], allowing for the creation of several parallel SMO models[19,36,37], reducing the total number of animals needed for the experiment. However, there are some disadvantages with parallel ischemia/reperfusion models in the same pig. Previous studies have shown that the cytokine levels are reduced when reperfusion of segments is continued in the same pig, due to increasing tolerance levels[64,65]. In addition, we observed periods of increasing heart rate, decreasing blood pressure, fever, and increasing permeability of the intestines, following the late reperfusion intervals. Increased heart rate, decreased blood pressure and fever may be systemic responses related to the release of increasing quantities of harmful substances following the late reperfusion intervals[66,67]. The increasing permeability[45] was visible as fluid droplets on the surface of the reperfused segments and increasing amounts of peritoneal fluid.

Inspection of the control tissue after 12 h gave an indication of the systematic effects on the surrounding perfused jejunum. LM showed mild reactive and inflammatory changes (Table 3), while TEM of the muscularis propria and serosa showed cells with some swollen mitochondria with vacuolated matrices. The microdialysis results and the histological grading systems did not indicate any changes in the control specimens. So, although some minor changes could be observed in the control intestine, we find it unlikely that this had any confounding effects on the outcome of the experiments. Thus, the observed ischemic changes in each occluded segment in the same pig are likely independent of systemic effects until the onset of reperfusion.

In conclusion, in the present porcine model with segmental occlusion of the jejunal mesentery, the intestinal tissue was judged to be probably irreversibly damaged when exposed to $\geqslant$ 4 h of ischemia and then reperfused. Using microdialysis to monitor intraluminal lactate and glycerol allowed us to closely monitor the onset and duration of ischemia, and the onset of reperfusion, but we were unable to find sufficient level of association between tissue viability and metabolic markers to be clinically relevant. The sequence of ischemia/reperfusion injury using the SMO model does not follow the outwards direction from the mucosa to the outer muscular layer, as most current histological grading and classification system suggest. Evaluation of intestinal viability based on return of color and the presence of peristalsis did not match well with histologic assessment of tissue viability.

ARTICLE HIGHLIGHTS

Research background

The clinical gold standard used on humans for assessment of intestinal viability is still based on palpation, visual inspection, bleeding from cut ends and the use of second look operations. The high mortality rates related to acute mesenteric ischemia have not been reduced drastically since the 1980's.

Research motivation

We are investigating methods to improve the accuracy of intraoperative surgical decision making with respect to assessment of the viability of ischemic/reperfused intestine. To assess the accuracy of these methods we need a reference for the limits of intestinal tissue viability. As the pathophysiology of ischemia/reperfusion in the porcine model is similar to humans, and because the pig model has been suggested as a reference standard in intestinal transplantation research, we decided to investigate the jejunal viability limit in a pig model. Our hypothesis is that the results with a pig model can have translational relevance for humans.

Research objectives

We investigated viability assessment in a porcine model of warm ischemia on jejunum with mesenteric occlusion, followed by reperfusion. Our aim was to determine the time point of irreversible damage, to provide a reference for experimental approaches to intestinal viability assessment.

Research methods

We created parallel segmental models on the jejunum in 15 pigs, by clamping the mesenteric arteries and veins for 1 to 16 h. Reperfusion was initiated after different intervals of ischemia (1-8 h) and subsequently monitored for 5-15 h. We compared the results from visual inspection with histology (light microscopy and transmission electron microscopy) and intraluminal microdialysis. The intestinal injury was graded using Park/Chiu and modified Swerdlow grading.

Research results

Only jejunal segments that had been ischemic for $\leqslant$ 3 h appeared viable (following $\geqslant$ 1 h of reperfusion). The jejunal segments that had been ischemic for 4 h showed (following $\geqslant$ 1 h of reperfusion) a total loss of crypt epithelium, extensive shrinkage and loss of myocytes in the outer layer of the muscularis propria. Intraluminal microdialysis allowed us to closely monitor the onset and duration of ischemia and the onset of reperfusion. We observed return of color and peristalsis in intestine that histologically contained areas of probably irreversible damage. The sequence of ischemia/reperfusion injury using the SMO model does not follow the outwards direction from the mucosa to the outer muscular layer, as most current histological grading and classification system suggest.

Research conclusions

In the present porcine model with segmental occlusion of the jejunal mesentery, the intestinal tissue was judged to be probably irreversibly damaged when exposed to $\geqslant$ 4 h of ischemia and then reperfused. Three hours of ischemia followed by reperfusion appeared to be the upper limit for viability in this model. We were unable to find sufficient level of association between tissue viability and metabolic markers to conclude that microdialysis is clinically relevant for viability assessment. Evaluation of color and motility appears to be poor indicators of intestinal viability.

Research perspectives

Segmental mesenteric occlusion provides reproducible injury in porcine jejunum and appears to be a relevant model for studies on viability assessment. Future studies should consider viability assessment in settings where the various etiologic factors related to acute mesenteric ischemia (emboli, arterial and venous thrombus and nonocclusive ischemia) can be evaluated, as good reference models are needed for each etiology.

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ORIGINAL ARTICLE

Clinical Trials Study

Quantitative assessment of hepatic fibrosis in chronic hepatitis B and C: T1 mapping on Gd-EOB-DTPA-enhanced liver magnetic resonance imaging

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Abstract

AIM

To assess the accuracy of Look-Locker on gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) for staging liver fibrosis in chronic hepatitis B/C (CHB/C).

METHODS

We prospectively included 109 patients with CHB or CHC who underwent a 3.0-Tesla MRI examination, including T1-weighted and Look-Locker sequences for T1 mapping. Hepatocyte fractions (HeF) and relaxation time reduction rate (RE) were measured for staging liver fibrosis. A receiver operating characteristic analysis using the area under the receiver operating characteristic curve (AUC) was used to compare the

diagnostic performance in predicting liver fibrosis between HeF and RE.

RESULTS

A total of 73 patients had both pathological results and MRI information. The number of patients in each fibrosis stage was evaluated semiquantitatively according to the METAVIR scoring system: F0, *n* = 23 (31.5%); F1, *n* = 19 (26.0%); F2, *n* = 13 (17.8%); F3, *n* = 6 (8.2%), and F4, *n* = 12 (16.4%). HeF by EOB enhancement imaging was significantly correlated with fibrosis stage (r = -0.808, P < 0.05). AUC values for diagnosis of any (≥ F1), significant (≥ F2) or advanced (≥ F3) fibrosis, and cirrhosis (F4) using HeF were 0.837 (0.733-0.913), 0.890 (0.795-0.951), 0.957 (0.881-0.990), and 0.957 (0.882-0.991), respectively. HeF measurement was more accurate than use of RE in establishing liver fibrosis staging, suggesting that calculation of HeF is a superior noninvasive liver fibrosis staging method.

CONCLUSION

A T1 mapping-based HeF method is an efficient diagnostic tool for the staging of liver fibrosis.

Key words: Liver fibrosis; Gd-EOB-DTPA; Look-Locker; Hepatocyte fraction; Liver function; Magnetic resonance imaging relative enhancement

Core tip: T1 mapping using the Look-Locker method with gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging at 3-Tesla by calculating the hepatocyte fraction is an efficient method for the assessment of liver fibrosis in patients with chronic hepatitis B and C, and this method is superior to using reduction rate.

Pan S, Wang XQ, Guo QY. Quantitative assessment of hepatic fibrosis in chronic hepatitis B and C: T1 mapping on Gd-EOB-DTPA-enhanced liver magnetic resonance imaging. *World J Gastroenterol* 2018; 24(18): 2024-2035 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/2024.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.2024

INTRODUCTION

Chronic hepatitis B (CHB) and chronic hepatitis C (CHC) are major health problems[1,2] and important factors globally in the development of hepatic fibrosis and even cirrhosis[3,4]. Liver fibrosis is a diffuse pathological change caused by chronic liver disease. As fibrosis progresses, it leads to cirrhosis and even cancer[5]. Early diagnosis and monitoring of liver fibrosis, and intervention with timely and effective treatments, are critical for patients with liver disease.

At present, liver biopsy is the gold standard for diagnosis of liver fibrosis. Tissue acquisition from a liver can be performed by endoscopic ultrasound-guided fine-needle aspiration[6-8], particularly for the left lobe[9,10]. This method is invasive, with a risk of bleeding and increased tissue injury, and repeatability of the examination is poor[11,12]. Therefore, noninvasive, comprehensive and accurate methods of diagnosing liver fibrosis are required. In recent years, noninvasive methods have been increasingly used, such as serological examination[13], ultrasound-based elastography[14], diffusion-weighted imaging (DWI)[15], magnetic resonance enterography (MRE)[16], T1 rho[17] and texture analysis[18].

Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is a liver-specific contrast agent that has a higher hepatocellular uptake rate than the traditionally used gadobenate dimeglumine (Gd-BOPTA)[19]. In previous studies, Gd-EOB-DTPA-enhanced magnetic resonance imaging (MRI) was mainly used to diagnose focal liver lesions, especially hepatocellular carcinomas, and even on functional MR cholangiography[20-22]. It has been shown that the intracellular transport mechanisms of Gd-EOB-DTPA are mediated by organic anion-transporting polypeptides (OATPs)[23]. Liver fibrosis obstructs the delivery of Gd-EOB-DTPA to hepatic cell surface transporters[24], contributing to a decline in OATP expression in the diseased liver[25], and consequently causing a decline in the T1-shortening effect of gadoxetic acid[26].

Recording liver parenchymal T1 values before and after drug administration allows the T1 relaxation time reduction rate (RE) to be calculated, reflecting the functional hepatocyte-specific uptake of gadoxetic acid and, thus, the state of the liver[27]. This method has been shown to have accurate diagnostic value in the assessment of liver fibrosis and is based on the traditional diagnostic method of Gd-EOB-DTPA-enhanced MR.

Use of T1 mapping technology is a noninvasive, quantitative method for determining tissue T1 relaxation time. After liver fibrosis, excessive accumulation of extracellular matrix proteins occur, leading to T1 relaxation time changes in fibrotic tissues. Therefore, T1 mapping is theoretically applicable to studies of liver fibrosis, such as the variable flip angle T1 mapping technique[28], which has been shown to be effective in diagnosing liver fibrosis.

T1 mapping using the Look-Locker method is one of the fastest, most efficient and reliable approaches to T1 quantification[29]. We proposed a method based on a simple pharmacokinetic model and ΔR1 values to calculate a hepatocyte fraction (HeF). In this method, changes to R1 in liver and spleen after EOB administration are calculated to obtain the HeF. In previous studies, the Look-Locker technique has mostly been used in the assessment of myocardial fibrosis and liver function[30]. However, its application in the clinical diagnosis of liver fibrosis has not yet been reported.

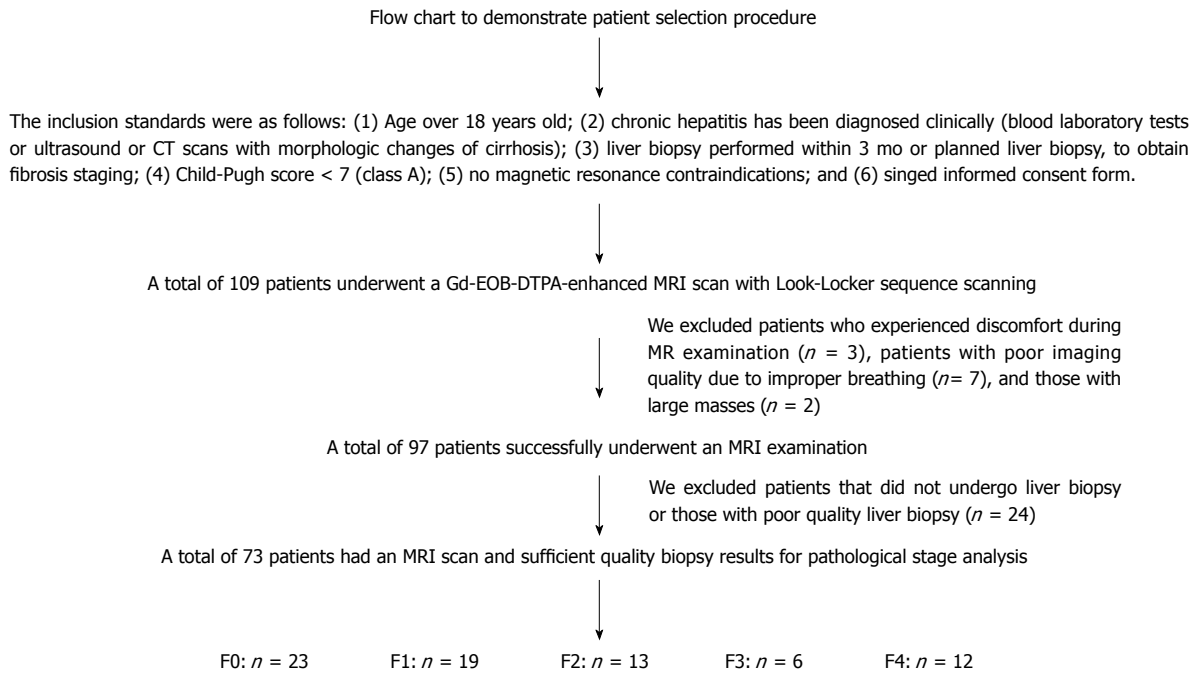

Figure 1 Flow chart to demonstrate the patient selection procedure.

Therefore, we proposed a working hypothesis that it was possible to diagnose liver fibrosis by calculating the HeF in the Look-Locker sequence by measuring Gd-EOB-DTPA-enhanced T1 signal intensity, and that this method would be superior to using RE. The purpose of this study, based on pathologic gold standards, was to quantitatively assess the level of hepatic fibrosis in hepatitis B and C patients by calculating the HeF and to compare the results with traditional T1-enhanced test parameters with the aim of establishing a novel noninvasive diagnosis method for liver fibrosis.

MATERIALS AND METHODS

This cross-sectional, prospective study was performed between August 2016 and June 2017. The study was approved by our institutional review board, and written informed consent was obtained from the participants prior to the study.

Patient population

The subjects of the study were patients with suspected or known chronic liver disease attending our hospital's infectious disease department. The inclusion criteria were as follows: (1) > 18-years-old; (2) chronic hepatitis diagnosed clinically (blood laboratory tests, ultrasound, or computed tomography scans showing morphological cirrhotic changes); (3) liver biopsy performed within 3 mo prior to the study or liver biopsy planned to obtain fibrosis staging; (4) Child-Pugh score < 7 (class A); (5) no MR contraindications; and (6) signed informed consent.

A total of 109 patients met the inclusion criteria, with 84 having CHB and 25 having CHC. We excluded 3 patients due to discomfort during the MR examination, 7 patients due to poor imaging quality from improper breathing, and 2 with a mass that was too large. The remaining patients (n = 97) were scheduled for a liver biopsy within 1 wk of the MRI. Of these 97, 20 did not undergo liver biopsy, and 4 were excluded due to poor liver biopsy quality. The standards for patient inclusion and exclusion are shown in Figure 1.

MRI acquisition

MR images were obtained with a 3-Tesla MRI system (Ingenia; Phillips Healthcare, Best, Netherlands) using a 32-channel torso phased-array coil. Patients were instructed to fast without water intake for 4-6 h before MR scanning. Before the examination, patients were trained to reduce breathing frequency or an abdominal binder was used to limit breathing frequency, to reduce interference during image acquisition.

A volume of 0.025 mmol/kg Gd-EOB-DTPA (Bayer Healthcare, Berlin, Germany) was administered at a rate of 1-2 mL/s. Following this, 30 mL saline was administered to flush the residual contrast reagent from the injection tube. T1WI and Look-Locker sequences were obtained twice (before and after the Gd-EOB-DTPA administration). To obtain T1 relaxation time, enhanced images were recorded 18 min after the Gd-EOB-DTPA injection.

The T1WI sequence was obtained using the scan parameters of FOV = 356 mm × 262 mm; slice thickness = 7 mm, 24 slices, in-plane resolution = 1.6 mm × 1.96 mm, matrix = 220 × 133, TR/TE = 12/2.3 ms, and band width = 361.9 kHz. Two-dimensional (2D) T1 maps were obtained using Look-Locker sequencing before and 20 min after the Gd-EOB-DTPA administration[31]. A three-

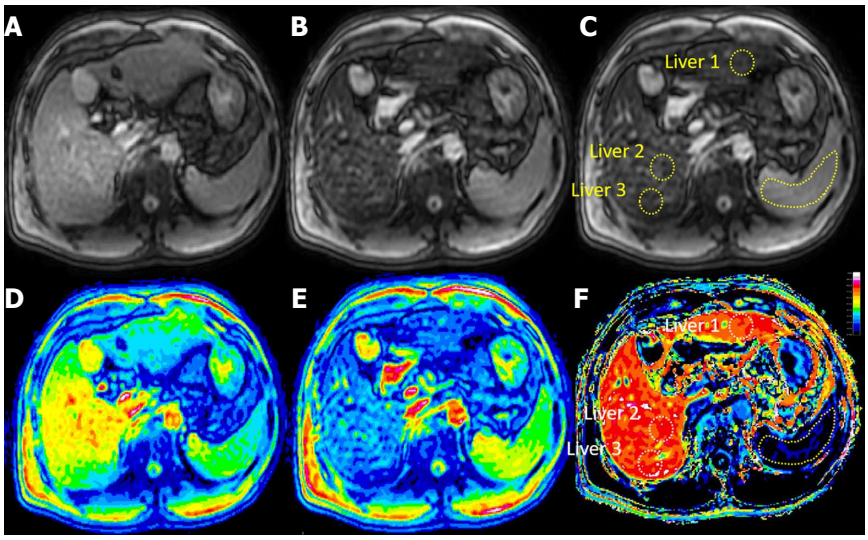

Figure 2 Precontrast (A, D) and postcontrast (B, E) T1 maps in a 72-year-old male with a METAVIR score of F4. The hand-drawn regions of interest of the liver and spleen are shown (C; dotted closed curves). HeF image (F) is shown, and HeF liver 1, HeF liver 2 and HeF liver 3 values were 68.13%, 72.46% and 70.45%, respectively, resulting in a HeF liver average of 70.34%. HeF: Hepatocyte fraction.

lead vector cardiogram was used for electrocardiogram gating. The T1 map was calculated from the Look-Locker sequence using the scan parameters of 2D image with single slice, TE/TR = 1/6 ms, 3.5 mm × 3.5 mm × 8 mm acquisition resolution, and 1.37 mm × 1.37 mm × 8 mm for recon, FA = 7°, two shots TFE with TFE factor 16, shot interval = 5 s for full T1 relaxation, SENSE factor = 2, and scan time = 15 s, with breath holding.

T1 maps were then automatically calculated using HepFract work-in-progress software (Phillips Healthcare).

Assessment of pathological specimens

Liver biopsy was performed under ultrasound guidance using an intercostal approach with a 14G disposable needle (MN1420; Bard Biopsy Systems, Tempe, AZ, United States) under local anesthesia. Liver specimens < 15 mm or containing < 11 portal tracts were excluded. Pathological sections of the biopsies were stained using the Masson method. Each pathologic section was read by two doctors with more than 10 years of pathologic diagnostic experience and who were unaware of the patient serological or imaging diagnosis. If the opinion of the two pathologists differed, a final diagnosis was reached by, or after, discussion with a more senior pathologist.

Fibrosis stage was evaluated semiquantitatively according to the METAVIR scoring system[32], with grading on a 5-point scale as follows: F0, no fibrosis; F1, fibrous portal expansion but without septa formation; F2, few bridges or septa; F3, numerous septa formation without cirrhosis; and F4, cirrhosis[33].

MRI analysis

As mentioned above, two doctors with more than 10 years of experience in the diagnosis of abdominal imaging - both of whom were unaware of the patient's laboratory results, pathologic grade or clinical diagnosis prior to measurement - assessed the MR images, with any inconsistency finalized by a senior clinician.

The scanned pre- and post Look-Locker images were saved in PAR-REC format, with the files imported into the HepFract processing software (Philips Scientific Software). Regions of interest (ROIs) were defined manually. A spleen ROI of about 4-5 cm^2 was drawn first, and three ROI of approximately 2-3 cm^2 were selected and marked in the liver parenchyma, two in the right lobe and three in the left (Figure 2), avoiding visible macroscopic vascular areas, the bile duct and the liver edge. No selection was made if the left lobe was too small or the image quality was insufficient. The HeF was calculated using the following formulas:

R1 change after EOB in both liver and spleen:

$$\Delta R1_{Liver} = 1 - \varphi_{Liver} \times \Delta R1_{Hepatobiliary} + \varphi_{Liver} \times \Delta R1_{BloodEES}$$

and

$$\Delta R1_{Spleen} = \varphi_{Spleen} \times \Delta R1_{BloodEES}$$

Where φ = total tissue water content [blood and extracellular space (EES)], φ_{Liver} = 0.23, and φ_{Spleen} = 0.3).

HeF:

$$(\Delta R1_{Hepatobiliary})/(\Delta R1_{Hepatobiliary} + \Delta R1_{BloodEES}) \times 100\ (\%),$$

Where $\Delta R1_{Hepatobiliary}$ = the T1 relaxation rate change of the liver parenchyma ROI before and after contrast reagent administration and $\Delta R1_{BloodEES}$ = the T1 relaxation rate change of blood, which was estimated based on the comparison of T1 relaxation rate of spleen parenchyma and liver parenchyma ROIs.

RE image processing was performed using DICOM Viewer R3.0 SP3 software (Philips). Images at the same Look-Locker level were preferred. Three ROIs were selected from the T1 liver images before and after Gd-EOB-DTPA administration. The ROIs were selected according to anatomical signs and the ROI positions of the Look-Locker images as far as possible. Figure 3 shows

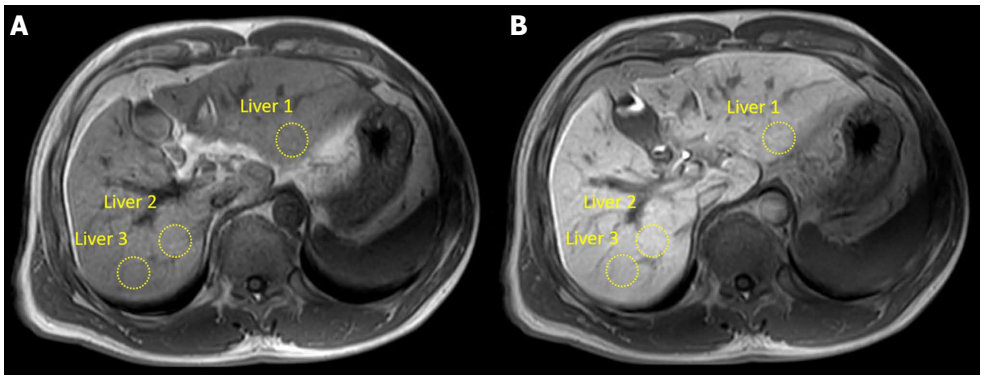

Figure 3 Precontrast (A) and postcontrast (B) T1-weighted images in a 72-year-old male with a METAVIR score of F4. The hand-drawn regions of interest of the liver are shown (dotted closed curves). RE was 0.45. RE: Reduction rate of T1 relaxation time.

Table 1 Patients' characteristics *n* %

Characteristic	No fibrosis, *n* = 23	Liver fibrosis, *n* = 50	All, *n* = 73
Age in yr	39.5 ± 11.1	41.3 ± 12.5	40.8 ± 12.1
Sex			
Male	17 (74)	30 (60)	47 (64)
Female	6 (26)	20 (40)	26 (36)
Height in m	1.72 ± 0.09	1.70 ± 1.21	1.71 ± 1.15
Weight in kg	77.21 ± 17.32	78.23 ± 16.12	78.10 ± 16.64
BMI in kg/m^2	24.09 ± 4.91	22.06 ± 4.57	23.73 ± 4.65
CHB	21 (34)	41 (66)	62 (85)
CHC	2 (18)	9 (82)	11 (15)

Data are presented as *n* (%) or mean ± SD. BMI: Body mass index; CHB: Chronic hepatitis B; CHC: Chronic hepatitis C.

ROI selection for the RE method. The RE was calculated according to the formula:

RE = [(Post-Pre)/Pre] × 100 (%),

Where Pre and Post were the average signal intensity of liver parenchyma ROIs before and after Gd-EOB-DTPA administration.

Finally, the averages of the HeF, RE, Post, and Pre calculations for the three ROIs were calculated.

Statistical analysis

After testing for normality with the Shapiro-Wilk test, HeF, RE, Post, and Pre were expressed as the mean ± standard deviation. One-way analysis of variance followed by Bonferroni's/Tamhane's T2 post-hoc comparison were performed to compare the means. Spearman's rank correlation coefficient (*r*) was used to show the correlation between HeF, RE, Post, Pre, and histological scores.

We performed receiver operating characteristic (ROC) analysis of the different stages of fibrosis and area under the ROC curve (AUC) analysis was used to evaluate the following classifications: F0 *vs* F1-F4 (≥ F1); F0-F1 *vs* F2-F4 (≥ F2); F0-F2 *vs* F3-F4 (≥ F3); and F0-F3 *vs* F4, using Pre, Post, the RE of the hepatobiliary phase and the HeF, based on Look-Locker. The optimal discrimination thresholds for RE and HeF were determined by maximizing the sums of sensitivity and specificity. The cut-off values, sensitivity, specificity, negative predictive value, positive predictive value, positive likelihood ratio and negative likelihood ratio were calculated. Comparisons of AUCs were carried out using the method proposed by DeLong *et al*[34].

Data analysis was performed with SPSS software, version 17.0 (SPSS, Chicago, IL, United States) and MedCalc version 7.4.2.0 (MedCalc Software, Mariakerke, Belgium) statistical software. A P value < 0.05 was considered significant.

RESULTS

Patient characteristics

The epidemiological characteristics of the enrolled patients, based on the presence or absence of fibrosis, are summarized in Table 1. We included a total of 73 patients [47 (64%) male and 26 (36%) female] who were eligible for inclusion on histopathological findings. Of these, 62 (85%) were infected with CHB and 11 (15%) were infected with CHC. Patient age ranged from 19-67 years (40.8 ± 12.1 years). Among them, there were 23 patients (31.5%) without fibrosis and 50 patients (68.5%) with fibrosis. Mean body mass index was 23.73 ± 4.65 kg/m^2 for the overall sample, and 15 of the 73 patients (21%) were overweight/obese (> 25 kg/m^2), 9 with CHB and 6 with CHC.

Differences in MRI at different levels of fibrosis

The average Post, RE and HeF (%) in patients without fibrosis was 356 ± 44, 0.94 ± 0.18 and 88.77 ± 5.10, respectively, which were significantly lower than in patients with fibrosis (Post: 331 ± 45; RE: 0.79 ± 0.23; HeF (%): 76.31 ± 11.23), and these values were significantly different between fibrotic and non-fibrotic patients (P = 0.037, 0.009 and < 0.001, respectively). Pre was not significantly different between patients with or without fibrosis (P = 0.235). The Pre, Post, RE and HeF (%) (mean ± SD) in patients with different grades of fibrosis are summarized in Table 2.

Post, RE and HeF were significantly different among

Table 2 Pre, Post, reduction rate of T1 relaxation time, and hepatocyte fraction (%) of patients at different METAVIR fibrosis stages

Fibrosis stage, (METAVIR)	F0, *n* = 23	F1, *n* = 19	F2, *n* = 13	F3, *n* = 6	F4, *n* = 12	Total, *n* = 73
Mean pre-T1 liver	180 ± 28	188 ± 19	174 ± 17	192 ± 22	200 ± 24	186 ± 24
Mean post-T1 liver	356 ± 44	355 ± 38	316 ± 34	336 ± 43	305 ± 50	338 ± 46
RE	0.94 ± 0.18	0.89 ± 0.20	0.94 ± 0.16	0.71 ± 0.17	0.52 ± 0.15	0.84 ± 0.23
HeF (%)	88.77 ± 5.10	84.23 ± 6.99	79.71 ± 8.09	70.90 ± 7.27	62.80 ± 7.01	80.24 ± 11.30

Data are presented as mean ± SD. HeF: Hepatocyte fraction; RE: Reduction rate of T1 relaxation time.

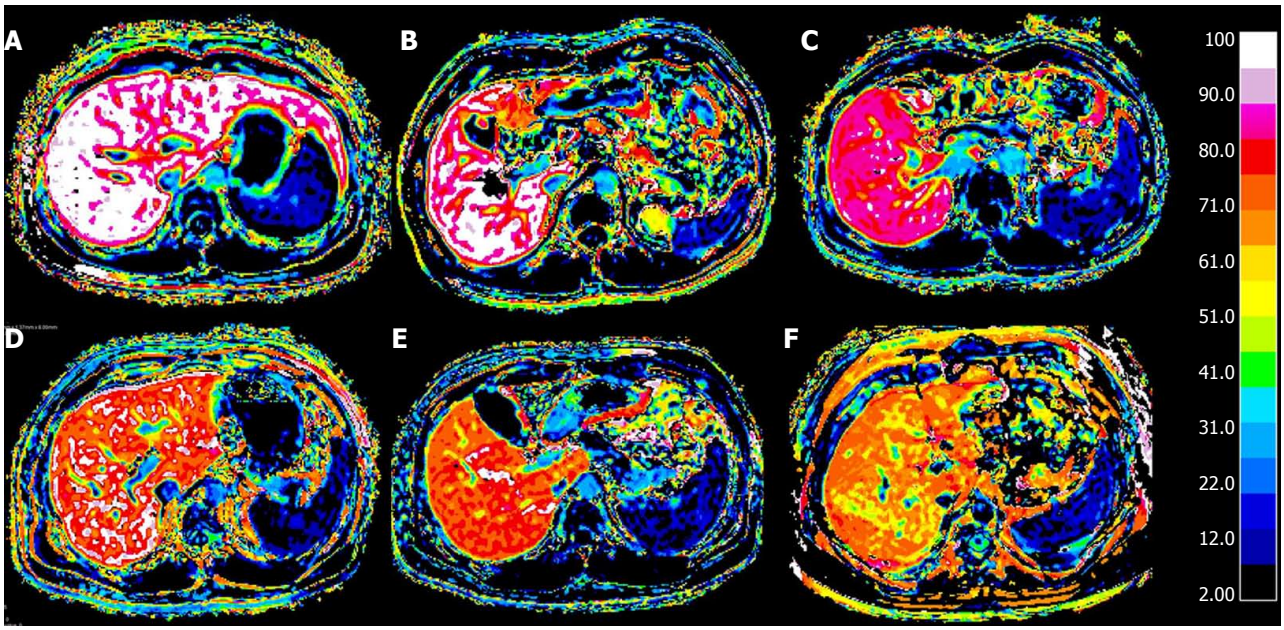

Figure 4 Representative images from patients with liver fibrosis at F0-F4 METAVIR stages. A: F0, HeF = 97.16%; B: F1, HeF = 89.21%; C: F2, HeF = 79.92%; D: F2, HeF = 72.94%; E: F3, HeF= 69.17%; F: F4, HeF = 62.43%. HeF: Hepatocyte fraction.

different grades of liver fibrosis (all $P < 0.05$). In the comparison of HeF in different groups of patients with different levels of fibrosis, each pair comparison was significantly different ($P < 0.001$), except for F1 and F2 (F1 *vs* F2 = 84.23 ± 6.99 *vs* 79.71 ± 8.09, $P = 0.065$). RE was not significantly different between the comparison groups F0 *vs* F1, F0 *vs* F2, and F1 *vs* F2 ($P = 0.365$, 0.490, and 0.912, respectively), but was significantly different between other comparison groups ($P < 0.05$). Post was statistically significantly different between patients with liver cirrhosis (F4) and patients without liver fibrosis (F0) (F4 *vs* F0 = 356 ± 44 *vs* 305 ± 50, $P < 0.001$), and was not significantly different between any other comparison groups ($P > 0.05$). Pre was not significantly different among different levels of liver fibrosis ($P > 0.05$). Figure 4 shows the HeF images in different stages of liver fibrosis.

Correlation analysis

RE and HeF were not correlated with body mass index or age (Spearman's correlation test, $r = 0.034$ and 0.247, $P = 0.847$ and 0.071, respectively), and did not differ between males and females (RE: 0.81 ± 0.27 *vs* 0.83 ± 0.16; HeF (%): 84.29 ± 8.17 *vs* 86.29 ± 6.10; both $P > 0.05$ for the independent samples *t*-test).

HeF and RE showed a strong correlation with fibrosis stage (RE: $r = -0.773$ (-0.852 to 0.661); HeF: $r = 0.808$ (-0.875 to 0.709); both $P < 0.001$). Post was moderately associated with grade of liver fibrosis [$r = -0.525$ (-0.674 to 0.336), $P < 0.001$]. Pre was not related to fibrosis grade [$r = 0.188$ (-0.045 to 0.4003), $P = 0.112$]. Correlation between Pre, Post, RE and HeF (%) with fibrosis ratings is summarized in Table 3 and Figure 5. RE was moderately correlated to HeF [$r = 0.539$ (0.353–0.684), $P < 0.001$].

ROC analysis

The AUC values, optimal cut-off values and the respective diagnostic performances for liver fibrosis measured by RE and HeF are summarized in Table 4 and Figure 6. The AUC values for HeF and RE were significantly higher than those for Pre and Post for detection of all fibrosis stages ($P < 0.05$). In the AUC comparison of HeF and RE, HeF had slightly higher AUCs than RE for discriminating ⩾ F1 (HeF *vs* RE = 0.837 *vs* 0.678, $P = 0.028$), ⩾ F2 (HeF *vs* RE = 0.890 *vs* 0.723, $P = 0.008$). HeF and RE for ⩾ F3 and F4 stage AUC showed no significant difference (HeF *vs* RE= 0.957 *vs* 0.921, $P = 0.418$; HeF *vs* RE = 0.957 *vs* 0.962,

Table 3 Correlation of reduction rate of T1 relaxation time, hepatocyte fraction, and METAVIR fibrosis stages

	Pre	Post	RE	HeF
r (95%CI)	0.188 (-0.045 to 0.4003)	-0.525 (-0.674 to 0.336)	-0.773 (-0.852 to 0.661)	-0.808 (-0.875 to 0.709)
P value	0.112	0.001	< 0.001	< 0.001

HeF: Hepatocyte fraction; RE: Reduction rate of T1 relaxation time.

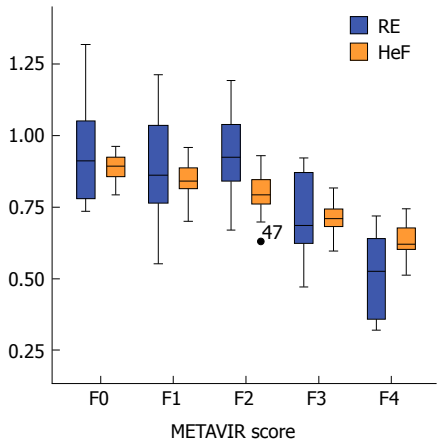

Figure 5 Box-and-whisker plots of T1 values. RE and HeF are shown for each METAVIR stage in relation to CHB and CHC. T1 values are reported on the Y-axis, and METAVIR stage of fibrosis is reported on the X-axis. CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; HeF: Hepatocyte fraction; RE: Reduction rate of T1 relaxation time.

respectively).

DISCUSSION

The results of our study indicated that T1 parameters from pre- or post-contrast T1 maps (HeF) and RE had good diagnostic value in the assessment of CHB, CHC and liver fibrosis. HeF and RE both had good diagnostic performance in advanced liver fibrosis and cirrhosis (⩾ F3 and F4) (AUC > 0.9). In diagnosis at ⩾ F1 and ⩾ F2 stages, HeF was better than RE.

Previous studies have used RE[35], liver-to-spleen ratio[36], contrast enhancement index[37] or dynamic measurements[38] based on Gd-EOB-DTPA-enhanced MRI to quantify parenchymal enhancement. In recent years, T1 mapping technology based on Gd-EOB-DTPA enhancement has been used mainly in the study of liver disease diagnosis. Katsube *et al*[39] first reported that evaluation of hepatic uptake of Gd-EOB-DTPA using T1 mapping of liver parenchyma could help estimate liver function. Kiyohisa *et al*[40] showed similar results in their study, which also demonstrated the diagnostic value of T1 mapping in liver disease.

At present, T1 mapping is used to evaluate the degree of hepatic fibrosis, but only a few studies have focused on liver fibrosis caused by chronic viral hepatitis (CHB, CHC). Li *et al*[28] found that in CCl_4-induced liver fibrosis in New Zealand rabbits, using the T1-mapping technique based on a series of liver acquisition volume acceleration sequences, AUCs in ⩾ F1, ⩾ F2 and ⩾ F3 stages from the ROC analysis were 0.803, 0.712 and 0.696, respectively. However, the study did not include F4 data.

Our HeF results were based on clinical patients, which have better reference values than animal experiments, and results for all the fibrosis stages were obtained. Yang *et al*[41] used 3D gradient-echo imaging on a 1.5-T MRI scanner to study volumetric interpolated breath-hold examination in liver fibrosis after CHB infection. They found that reduction in T1 relaxation time 20 min after gadoxetic acid injection (ΔT1, ΔR1%) compared with before injection and the contrast uptake rate (K_{Hep}) decreased significantly as the fibrosis score increased. In that study, ΔR1% had the highest correlation with fibrosis stage (r = -0.626), followed by K_{Hep} (r = -0.527), and ΔT1 (r = 0.513).

The above mentioned studies were based on 1.5-T MR, while our images were acquired using 3.0-T MR with better image quality, assisting in image analysis and processing. Banerjee *et al*[42] explored the relationship between corrected T1 parameter (cT1) and hepatic fibrosis Ishak rank, based on a shortened modified Look-Locker inversion (known as shMOLLI) recovery sequence T1 mapping technique, and found that cT1 was strongly correlated with increased liver fibrosis (cT1 *vs* Ishak [n = 84, r = 0.68], AUC of F ⩾ 1 stage = 0.94). While the AUC values for the most of the fibrosis groups were similar to those of our study, the correlation reported by Banerjee *et al*[42] was stronger than what was observed in this study where the AUC value for F ⩾ 1.

Banerjee's study enrolled a total of 84 patients. While the causes of liver fibrosis in their study were from different types of chronic liver disease, 31 cases were caused by virus[42]. A lack of research into viral hepatitis highlights the importance of studying changes in the degree of liver fibrosis caused by different types of liver disease. Sheng *et al*[43] have compared T1 mapping with RE on Gd-EOB-DTPA-enhanced MRI in the field of liver fibrosis assessment. The result showed that Gd-EOB-DTPA-enhanced T1 mapping might provide a reliable diagnostic tool in staging liver fibrosis, whereas it was research performed on rabbits[43]. As our study applies to human, having clinical significance that is superior to animal studies.

Among the T1 mapping techniques, Look-Locker has several advantages. It is efficient compared with conventional techniques, which have only sample one

Table 4 Performance of the mean reduction rate of T1 relaxation time and hepatocyte fraction for the prediction of METAVIR fibrosis stages according to cut-off values

Parameters	⩾ F1	⩾ F2	⩾ F3	= F4
Cut-off				
HeF (%)	82.93	79.24	74.37	74.37
RE	0.75	0.73	0.72	0.72
AUC				
HeF (%)	0.837 (0.733-0.913)	0.890 (0.795-0.951)	0.957 (0.881-0.990)	0.957 (0.882-0.991)
RE	0.678 (0.559-0.783)	0.723 (0.606-0.821)	0.921 (0.834-0.971)	0.962 (0.889-0.993)
Sensitivity, %				
HeF(%)	72.0 (57.5-83.8)	77.4 (58.9-90.4)	94.4 (72.7-99.9)	100.0 (73.5-100.0)
RE	44.0 (30.0-58.7)	58.1 (39.1-75.5)	88.9 (65.3-98.6)	100.0 (73.5-100.0)
Specificity, %				
HeF (%)	82.6 (61.2-95.0)	92.9 (80.5-98.5)	92.7 (82.4-98.0)	85.3 (73.8-93.0)
RE	100.0 (85.2-100.0)	92.9 (80.5-98.5)	92.7 (82.4-98.0)	86.9 (75.8-94.2)
PPV, %				
HeF (%)	90.0 (76.3-97.2)	88.9 (70.8-97.6)	81.0 (58.1-94.6)	57.1 (34.0-78.2)
RE	100.0 (84.6-100.0)	85.7 (63.7-97.0)	80.0 (56.3-94.3)	60.0 (36.1-80.9)
NPV, %				
HeF (%)	57.6 (39.2-74.5)	84.8 (71.1-93.7)	98.1 (89.7-100.0)	100.0 (93.2-100.0)
RE	45.1 (31.1-59.7)	75.0 (61.1-86.0)	96.2 (87.0-99.5)	100.0 (93.3-100.0)
PLR				
HeF (%)	4.14 (1.7-10.3)	10.84 (3.6-32.8)	12.99 (5.0-33.6)	6.78 (3.7-12.4)
RE	_	8.13 (2.6-25.2)	12.22 (4.7-31.8)	7.62 (4.0-14.5)
NLR				
HeF (%)	0.34 (0.2-0.5)	0.24 (0.1-0.5)	0.060 (0.009-0.4)	_
RE	0.56 (0.4-0.7)	0.45 (0.3-0.7)	0.12 (0.03-0.4)	_

AUC: Area under the curve; HeF: Hepatocyte fraction; NLR: Negative likelihood ratio; NPV: Negative predictive value; PLR: Positive likelihood ratio; PPV: Positive predictive value; RE: Reduction rate of T1 relaxation time.

point for each inversion pulse. In addition, Look-Locker is less sensitive to B1 heterogeneity and less prone to error compared with the variable flip angle method[44,45].

Some research into the relationship between RE and liver fibrosis has been conducted. The earliest discussion of the relationship between relative T1 values and fibrosis was reported by Smith *et al*[46] in 1981. However, subsequent studies did not confirm the correlations[47]. Verloh *et al*[26], in a study of the relationship between RE and liver fibrosis, found strong correlations between the uptake characteristics of Gd-EOB-DTPA with RE and the grade of fibrosis/cirrhosis, classified using the Ishak scoring system. The inclusion criteria for this experiment did not limit the type of chronic liver disease that led to liver fibrosis.

Feier *et al*[35] showed a strong correlation between the RE and METAVIR score (r = -0.65), which was consistent with our results (r = -0.773). Among their results, the AUC in the ⩾ F1 and ⩾ F2 stages was higher than our results (⩾ F1: 0.81 *vs* 0.68; ⩾ F2: 0.82 *vs* 0.72). The reasons for this difference in results are as follows. First, Feier *et al*[35] did not focus on liver fibrosis caused by chronic viral hepatitis (CHB, CHC); instead, they included patients with alcoholic liver disease and autoimmune hepatitis leading to liver fibrosis. Second, in our study, the F3 and F4 groups were small, which could have led to bias. Third, RE measurement is relatively simple and direct; however, some disadvantages are also obvious. MRI signal intensity is influenced by many factors, and the collection method of the image may make statistical analysis difficult. Look-Locker sequencing ensures consistent image acquirement and analysis. T1 maps obtained using Look-Locker sequencing may be more robust than simple signal intensity measurements on the hepatobiliary phase[48], as T1 maps are less affected by MR parameters at the same magnetic field strength than signal intensity measurements.

In contrast to the current international consensus on the diagnosis of liver fibrosis using MRE technology, our results showed that, based on Gd-EOB-DTPA-enhanced T1 mapping technology, HeF and METAVIR classification of liver fibrosis were significantly correlated (r = -0.808), although slightly lower than with the MRE technique (r = 0.899). Comparing the AUCs of the ⩾ F1, ⩾ F2, ⩾ F3 and F4 groups, the AUCs for MRE were 0.84, 0.88, 0.93 and 0.92, respectively[49]. Our HeF results were consistent with previous studies.

Compared with MRE technology, which requires a special hardware installation, Gd-EOB-DTPA-enhanced Look-Locker scanning can be operated on standard clinically used MR equipment, an important advantage making it a popular choice. In contrast, the results of studies using the apparent diffusion coefficient (ADC) value in conjunction with DWI to assess hepatic fibrosis were quite different, and the use of ADC values in diagnosis remains controversial. For example, Tokgöz *et al*[50] showed that ADC values in the different grades of fibrosis were not significantly different. A meta-analysis of DWI studies analyzing the use of ADC in

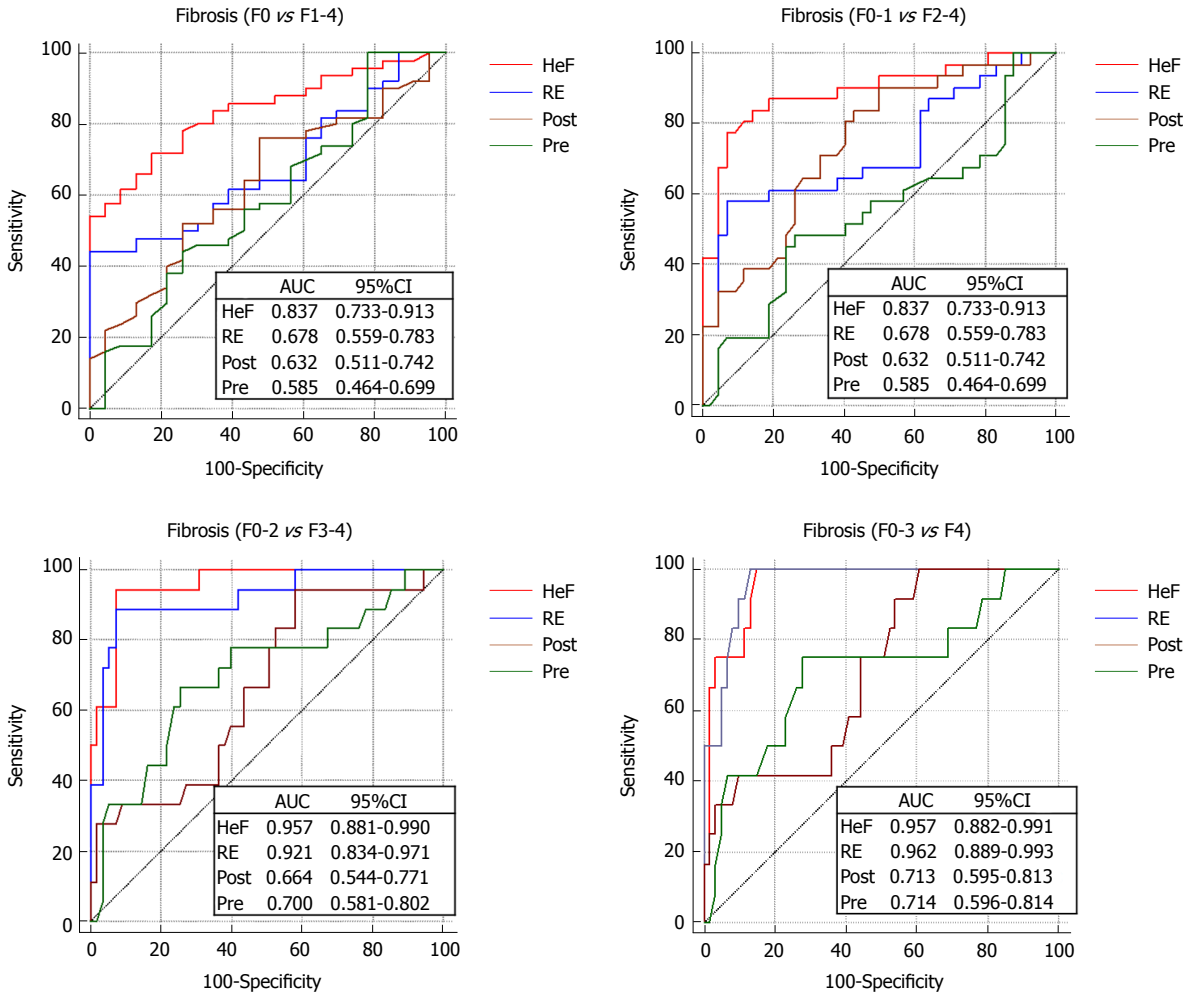

Figure 6 Comparison of the receiver operating characteristic curves of Pre, Post, RE and HeF for different fibrosis thresholds. From left to right: F0 *vs* F1-F4 (≥ F1), F0-F1 *vs* F2-F4 (≥ F2), F0-F2 *vs* F3-F4 (≥ F3), and F0-F3 *vs* F4 (≥ F4). The numbers in the boxes indicate the AUC values and 95%CIs. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; HeF: Hepatocyte fraction; RE: Reduction rate of T1 relaxation time.

liver fibrosis staging included a cumulative total of 613 patients in 10 studies[51]. It reported an AUC of 0.86 for F ≥ 1, 0.83 for F ≥ 2, and 0.86 for F ≥ 3, and concluded that DWI had a good diagnostic value for degree of liver fibrosis. Ding *et al*[52] compared DWI with Gd-EOB-DTPA-enhanced RE in the diagnosis of liver fibrosis; the results showed that RE was better than ADC. In our experiments, HeF was of superior diagnostic value than RE, so we can predict that HeF for liver fibrosis staging is of greater diagnostic value than DWI. Because different b values affect the results of ADC, they cannot be compared between studies because liver fibrosis staging is difficult to establish.

T1 mapping of gadoxetic acid-enhanced MR images using Look-Locker sequencing can be achieved using breath-holding and simplifying the image processing. We believe that this practical method has potential in the quantitative estimate of liver fibrosis and can be used as an important complementary sequence in clinical Gd-EOB-DTPA-enhanced MRI in patients with chronic liver disease.

Our study had some limitations. First, the sample size of this study was small, especially for the F3 stage category. Compared to similar studies which have shown inconsistency in hepatocellular function in the F ≥ 3 stage group (AUC: 0.63, 0.85, 0.87 and 0.93)[53], a larger sample size was needed for the F3 stage category. Second, the study only examined the information obtained at 20 min after Gd-EOB-DTPA administration, and did not analyze HeF measured at other times, such as 5 or 10 min. Third, the Look-Locker sequence was a 2D sequence that did not contain information about the whole liver. When the HeF scan is part of an examination, an enhanced sequence of the whole liver should be added to the scanning protocol because of its value in clinical diagnosis. Furthermore, mismatches between pre- and post-contrast images were observed due to motion and the long gap between scans. To improve accessibility for future clinical use, further development in fast multislice or 3D volume quantitative T1 mapping is needed with liver-specific motion registration.

In conclusion, this study showed a strong correlation between HeF and liver fibrosis stage in CHB and CHC.

Although HeF and RE are used to generate quantitative measurements to distinguish between different grades of liver fibrosis, HeF performed better than RE. This study showed that the T1 mapping-based HeF method is an efficient diagnostic tool for the staging of liver fibrosis.

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis B/C (CHB/C) are both leading causes of liver-related morbidity and mortality and predisposes patients to liver fibrosis, the excessive accumulation of extracellular matrix proteins. As fibrosis progresses, it leads to cirrhosis and even cancer. Early diagnosis and monitoring of liver fibrosis, and intervention with timely and effective treatments, are critical for patients with liver disease. At present, liver biopsy is the gold standard for diagnosis of liver fibrosis. Invasive methods have a risk of bleeding and increased tissue injury, and the repeatability of the examination is poor. Therefore, noninvasive, comprehensive and accurate methods of diagnosing liver fibrosis are required.

Research motivation

Currently, noninvasive methods have been increasingly used, such as serological examination, ultrasound-based elastography, diffusion-weighted imaging, magnetic resonance enterography, and texture analysis. None of these methods can replace the biopsy. T1 mapping *via* the Look-Locker method is one of the fastest approaches to T1 quantification, and is the most time efficient method for T1 mapping and less affected by magnetic resonance parameters than other methods. We proposed a method based on a simple pharmacokinetic model and ΔR1 values to calculate a hepatocyte fraction (HeF). Furthermore, mismatches between pre- and postcontrast images were observed due to motion and the long gap between scans. To improve accessibility for future clinical use, further development in fast multislice or 3D volume quantitative T1 mapping is needed with liver-specific motion registration.

Research objectives

We aimed to quantitatively assess the level of hepatic fibrosis in hepatitis B and C patients by calculating the HeF and compare the results with traditional T1-enhanced test parameters. In the future, more imaging methods should be compared with HeF, such as magnetic resonance enterography and diffusion-weighted imaging.

Research methods

One hundred and nine patients were included in the study. Magnetic resonance images were obtained with a gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced 3-Tesla magnetic resonance imaging system, including T1-weighted and Look-Locker sequences for T1 mapping. HeF and relaxation time reduction rate (RE) were calculated for staging hepatic fibrosis. Area under the receiver operating characteristic curve (AUC) was used to compare the diagnostic performance in predicting liver fibrosis between HeF and RE.

Research results

We included a total of 73 patients who were deemed eligible for inclusion on histopathological findings. The results of our study indicated that T1 parameters from pre- or postcontrast T1 maps (HeF) and RE had good diagnostic value in the assessment of CHB, CHC and liver fibrosis. HeF and RE both had good diagnostic performance in advanced liver fibrosis and cirrhosis ($\geqslant$ F3 and F4) (AUC > 0.9). In diagnosis at $\geqslant$ F1 and $\geqslant$ F2 stages, HeF was better than RE.

Research conclusions

This study showed a strong correlation between HeF and liver fibrosis stage in CHB and CHC. The methods use HeF and RE to generate quantitative measurements to distinguish different grades of liver fibrosis, but HeF performed better than RE. This study showed that the T1 mapping-based HeF method is an efficient diagnostic tool for the staging of liver fibrosis.

Research perspectives

Due to the limited number of patients included, further studies are needed to assess the performance of the HeF in hepatic fibrosis. More imaging methods should be compared in the field of liver fibrosis diagnosis.

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ORIGINAL ARTICLE

Observational Study

Thiopurines are negatively associated with anthropometric parameters in pediatric Crohn's disease

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Abstract

AIM

To determine the distribution of anthropometric parameter (AP)-*z*-scores and characterize associations between medications/serum biomarkers and AP-*z*-scores in pediatric Crohn's disease (CD).

METHODS

CD patients [< chronological age (CA) 21 years] were enrolled in a cross-sectional study. Descriptive statistics were generated for participants' demographic characteristics and key variables of interest. Paired *t*-tests were used to compare AP-*z*-scores calculated based on CA (CA *z*-scores) and bone age (BA) (BA *z*-scores) for interpretation of AP's. Linear regression was utilized to examine associations between medications and serum biomarkers with AP-*z*-scores calculated based on CA (n = 82) and BA (n = 49). We reported regression coefficients as well as their corresponding p-values and 95% confidence intervals.

RESULTS

Mean CA at the time of the study visit was 15.3 ± 3.5 (SD; range = 4.8-20.7) years. Mean triceps skinfold (P = 0.039), subscapular skinfold (P = 0.002) and mid-arm circumference (MAC) (P = 0.001) BA *z*-scores were higher than corresponding CA *z*-scores. Medications were positively associated with subscapular skinfold [adalimumab (P = 0.018) and methotrexate (P = 0.027)] and BMI CA *z*-scores [adalimumab (P = 0.029)]. Azathioprine/6-mercaptopurine were negatively associated with MAC (P = 0.045), subscapular skinfold (P = 0.014), weight (P = 0.002) and BMI (P = 0.013) CA *z*-scores. ESR, CRP, and WBC count were negatively associated, while albumin and IGF-1 BA *z*-scores were positively associated, with specific AP *z*-scores (P < 0.05). Mean height CA *z*-scores were higher in females, not males, treated with infliximab (P = 0.038). Hemoglobin (P = 0.018) was positively associated, while platelets (P = 0.005), ESR (P = 0.003) and CRP (P = 0.039) were negatively associated with height CA *z*-scores in males, not females.

CONCLUSION

Our results suggest poor efficacy of thiopurines and a possible sex difference in statural growth response to infliximab in pediatric CD. Prospective longitudinal studies are required.

Key words: Inflammatory bowel disease; Azathioprine/6-mercaptopurine; Biologics; Nutrition

Core tip: Azathioprine/6-mercaptopurine were negatively associated with specific anthropometric parameters, suggesting a possible negative effect *vs* poor efficacy of thiopurines in pediatric Crohn's disease (CD). Infliximab was positively associated with standardized height in females only, suggesting a possible sex difference in response to infliximab from the standpoint of statural growth in pediatric CD. Specific serum biomarkers were associated with standardized height in males only, supporting that inflammation has a more detrimental effect on statural growth in males with pediatric CD.

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INTRODUCTION

Several studies document alterations in anthropometric parameters in pediatric Crohn's disease (CD) such as lean mass deficits[1-5], reductions in fat free mass[6,7], fat mass deficits[3,5,7], low body mass index (BMI)[1-3,5-8], high BMI[7,8], and low height[1-3,5,9,10]. Similar to impaired statural growth (height velocity), a dynamic marker of disease status, body composition deficits may reflect poorly controlled disease despite the absence of overt clinical intestinal symptoms.

Delayed bone age (BA) is common in pediatric CD[10-16]. BA assessed by left hand X-ray is regarded as a valid measure of skeletal maturity[13-14,17-19]. Determination of BA allows clinically meaningful interpretation of growth in the context of skeletal maturity in pediatric CD[11]. Mean height, weight and BMI *z*-scores calculated based on BA (BA *z*-scores) are higher than corresponding *z*-scores calculated based on chronological age (CA) (CA *z*-scores) in pediatric CD[11].

The impact of accounting for BA in the interpretation of body composition is unclear. Accurate interpretation of body composition is important since it reflects nutritional[4] and disease status. Not only is nutritional status an important determinant of pubertal development and growth velocity[20], it is a prognostic factor for disease course[21-28]. Several factors affect nutritional status, including inflammation, medications, nutrient intake, and hormones[4,29,30]. The association between medications and serum inflammatory and hormonal biomarkers with anthropometric measurements is not well delineated in pediatric CD, particularly after adjusting for maturational status (BA).

Nutritional status is an important factor to consider when making therapeutic decisions given its association with poor outcomes[21-28]. Yet, the impact of treatments

on anthropometric measurements is poorly defined and has not received sufficient attention[31]. While there are well-documented sex differences in risk for statural growth impairment[9-10,14,21,32-35], sex differences in nutritional status require further study. Data regarding the relationship between medications and serum biomarkers with anthropometric parameters by sex, an important biological variable, are lacking.

Here we assessed body composition by skinfold measurements in pediatric CD. Our aims were to (1) determine the distribution of anthropometric parameters based on CA (CA *z*-scores) and BA (BA *z*-scores); and (2) characterize the associations between medications and serum biomarkers with anthropometric parameter *z*-scores in pediatric CD.

MATERIALS AND METHODS

Pediatric CD patients < CA 21 years enrolled in this cross-sectional study at University of California, San Francisco (UCSF) between January 2007 and July 2009 as previously described[10-11,36]. We excluded patients who received growth hormone ever or corticosteroids within 2 mo prior to study participation since more recent use would suppress the somatotropic axis and interfere with accurate assessment of insulin-like growth factor-1 (IGF-1) levels. Eighty-two patients completed the study.

Mid-arm circumference measurements and skinfold thickness measurements were collected to the nearest 0.1 mm from the non-dominant side of the body in triplicate and averaged. A measuring tape was used for mid-arm circumference measurements and Lange skinfold calipers were used for skinfold thickness measurements. The mid-arm circumference measurement was obtained at the mid-point between the olecranon process and acromion. The triceps skinfold measurement was obtained at the mid-point of the upper arm, halfway between the acromion and the olecranon. The subscapular skinfold was measured at a 45° angle just below the inferior angle of the scapula. One of two registered dietitians obtained the measurements. Both were trained using standardized NHANES methodologies with established inter-rater reliability[37]. Weight and height were measured using a digital scale (Scale-Tronix, White Plains, NY, United States) to the nearest 0.1 kg and stadiometer (Proscale, Accurate Technology, Inc., Cincinnati, OH, United States) to the nearest 0.1 cm, respectively. Body mass index (BMI) was calculated as the weight in kg divided by the square of the height in meters. Self-Tanner staging was performed[38]. Left hand x-rays obtained for BA were blindly interpreted by RL using the standards of Greulich and Pyle[17].

Medications of interest included adalimumab, 5-aminosalicylates, antibiotics, azathioprine/6-mercaptopurine (thiopurines), infliximab, and methotrexate.

We classified disease location as esophagus or stomach; small bowel, no colon; small bowel and colon; colon, no small bowel; perianal.

A lab draw was performed to measure serum IGF-1, insulin-like growth factor binding protein 3 (IGFBP-3), testosterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), albumin, alkaline phosphatase, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), hemoglobin, platelets, and white blood cell (WBC) count. Tubes for serum hormone levels and routine clinical labs were processed by Esoterix Endocrinology (Calabasas Hills, CA, United States) and UCSF clinical lab, respectively. Clinical information was collected.

Statistical analysis

We calculated CA *z*-scores for IGF-1, IGFBP-3, estradiol, testosterone, FSH, LH, mid-arm circumference, triceps skinfold, subscapular skinfold, weight, height, and BMI using reference values based in part on CA. Because pubertal growth acceleration correlates more closely with BA than CA[39], we also calculated BA *z*-scores for all 17 females ≤ CA 15 and 32 males ≤ CA 17 years, as epiphyses close at BA 15 in females and 17 years in males. We excluded all females > CA 15 and males > CA 17 years from BA analyses because sufficient reference data on variability of BA beyond these CA thresholds are not available. We transformed mid-arm circumference, triceps skinfold, subscapular skinfold, weight, height, and BMI measurements to *z*-scores[40-45]. Means and standard deviations (SDs) provided by Esoterix Endocrinology were used to calculate IGF-1 and IGFBP-3 *z*-scores. The mean and the upper and lower bounds of the normal ranges (specific to sex, age, and Tanner stage), accounting for asymmetry about the mean if present, were used to compute SDs for gonadotropins and sex hormones. Low and high *z*-scores are defined as *z*-scores < -2.0 and > 2.0, respectively.

Descriptive statistics were generated for participants' demographic characteristics and key variables of interest. Paired t-tests were used to compare CA *z*-scores and BA *z*-scores for interpretation of anthropometric parameters. We employed linear regression to assess the associations between predictors (medications and serum biomarkers) and outcomes (anthropometric parameter CA *z*-scores and BA *z*-scores). For outcomes based on CA *z*-scores (n = 82), we also conducted analyses including CA at study visit, sex, CRP, albumin, ESR, and hemoglobin in the model to adjust for potential confounding. We conducted additional analyses adjusting for disease activity indices, disease duration, stricturing disease and penetrating disease in these models. We analyzed height CA *z*-scores separately by sex because of well-established sex differences in risk for statural growth impairment[9-10,14,21,32-35]. We reported regression coefficients as well as their corresponding p-values and 95% confidence intervals (CI); P-values < 0.05 were considered as statistically significant. Data were analyzed using IBM SPSS Statistics 23.

Ethical considerations

We obtained Institutional Review Board Approval for the study protocol. Informed consent/assent were obtained

Table 1 Demographics, Tanner stage, disease location, and medications

Item	*n* (%)
Race	
Asian	12 (14.6)
East Asian	6
South Asian	6
Black/African American	1 (1.2)
Other	4 (4.9)
White	65 (79.3)
Ethnicity	
Hispanic or Latino	7 (8.5)
Not Hispanic or Latino	75 (91.5)
Tanner stage	
1	8 (9.8)
2	15 (18.3)
3	16 (19.5)
4	24 (29.3)
5	19 (23.2)
Disease location	
Esophagus or stomach	9 (11)
Small bowel, no colon	12 (14.6)
Colon, no small bowel	17 (20.7)
Small bowel and colon	53 (64.6)
Perianal disease	49 (59.8)
Medication	
Adalimumab	4 (4.9)
5-Aminosalicylates	50 (61.0)
Antibiotics	14 (17.1)
Azathioprine/6-Mercaptopurine	44 (53.7)
Infliximab	20 (24.4)
Methotrexate	7 (8.5)
Steroids ever	55 (67.1)

from parents/patients.

RESULTS

Participant characteristics

82 patients completed the study; 35 (43%) were female[10]. Mean CA at the time of the study visit was 15.3 ± 3.5 (SD; range = 4.8-20.7) years[10]. Mean CA at the time of inflammatory bowel disease (IBD) diagnosis was 12.1 ± 3.8 (0.5-17.9) years. Mean time since IBD diagnosis was 3.4 ± 2.8 (0.01-12.0) years. Race/ethnicity, Tanner stage, disease location, and medications are summarized in Table 1. History of corticosteroid use did not differ by sex[10].

Monotherapy vs combination therapy

Of the 44 patients on azathioprine/6-mercaptopurine, 33 (75%) were on thiopurine monotherapy, 10 (23%) were on combination therapy with infliximab and 1 (2%) was on combination therapy with adalimumab.

Of the 7 patients on methotrexate, 3 (43%) were on monotherapy, 2 (28.5%) were on combination therapy with infliximab and 2 (28.5%) were on combination therapy with adalimumab.

Of the 20 patients on infliximab, 8 (40%) were on monotherapy, 10 (50%) were on combination therapy with thiopurines and 2 (10%) were on combination therapy with methotrexate.

Of the 4 patients on adalimumab, 1 (25%) was on monotherapy, 1 (25%) was on combination therapy with thiopurines, 2 (50%) were on combination therapy with methotrexate.

Anthropometric parameters

Anthropometric parameters are summarized in Table 2.

Bone age vs chronological age for the interpretation of anthropometric parameters

For the 49 patients qualifying for BA analyses, mean BA (12.2 ± 2.9 years) was significantly lower than mean CA (13.1 ± 2.6 years) ($P < 0.0001$)[10]. Mid-arm circumference (0.35 units, 95%CI: 0.14-0.55; $P = 0.001$), subscapular skinfold (0.10 units, 95%CI: 0.04-0.16; $P = 0.002$), and triceps skinfold (0.05 units, 95%CI: 0.003-0.11; $P = 0.039$) BA *z*-scores were systematically higher than corresponding CA *z*-scores.

Medications, serum biomarkers, and anthropometric parameters

Tables 3 and 4 show the unadjusted and adjusted associations, respectively, between medication treatment, serum biomarkers and anthropometric parameter CA *z*-scores (height CA *z*-scores presented separately) that achieved statistical significance. Infliximab was not statistically significantly associated with mid-arm circumference, triceps skinfold, subscapular skinfold, weight or BMI CA *z*-scores (data not shown). Results did not change when disease activity indices, disease duration, stricturing disease or penetrating disease were included in the adjusted models.

Table 5 shows the unadjusted associations between serum biomarkers and anthropometric parameter BA *z*-scores (height BA *z*-scores presented separately). Medication treatments were not statistically significantly associated with anthropometric parameter BA *z*-scores.

Table 6 shows the unadjusted associations between medications, serum biomarkers and height CA *z*-scores by sex.

Table 7 shows the unadjusted association between serum biomarkers and height BA *z*-scores. Medications were not statistically significantly associated with height BA *z*-scores.

DISCUSSION

In our prospective, cross-sectional study, azathioprine/6-mercaptopurine were negatively associated with lean tissue mass (mid-arm circumference CA *z*-scores) and fat store (subscapular CA *z*-scores) measurements, and weight CA *z*-scores and BMI CA *z*-scores in pediatric CD. We previously reported thiopurine treatment was associated with lower standardized BA results[11]. From a mechanistic perspective, it is unlikely these associations represents a direct negative impact of thiopurines on skeletal maturation or anthropometric parameters. When examining the association between

Table 2 Summary of anthropometric parameters

Variable (*n*)	Mean ± SD	Range	Percent with *z*-scores > 2 (%)	Percent with *z*-scores < -2 (%)
Mid-arm circumference-CA-*z*-score *n* = 82	-0.64 ± 1.39	-5.03 to 2.88	2	17
Mid-arm circumference-BA-*z*-score *n* = 49	-0.54 ± 1.30	-2.86 to 2.50	2	16
Subscapular skinfold-CA-*z*-score *n* = 81	0.59 ± 0.83	-1.56 to 2.31	3	0
Subscapular skinfold-BA-*z*-score *n* = 48	0.64 ± 0.87	-1.17 to 2.27	4	0
Triceps-CA-*z*-score *n* = 81	1.02 ± 0.74	-0.88 to 2.79	11	0
Triceps-BA-*z*-score *n* = 49	1.10 ± 0.72	-1.17 to 2.47	8	0
Height-CA-*z*-score *n* = 82	-0.30 ± 1.02	-2.74 to 2.34	1	6
Height-BA-*z*-score *n* = 49	0.17 ± 1.12	-3.29 to 2.53	4	2
Weight CA-*z*-score *n* = 82	-0.17 ± 1.10	-3.49 to 2.20	4	5
Weight BA-*z*-score *n* = 49	0.11 ± 0.91	-2.52 to 1.97	0	2
BMI-CA-*z*-score *n* = 82	-0.07 ± 1.04	-2.78 to 2.17	4	4
BMI BA-*z*-score *n* = 49	0.05 ± 0.86	-2.58 to 2.09	2	2

BA *z*-score: *z* score based on bone age; BMI: Body mass index; CA *z*-score: *z* score based on chronological age.

Table 3 Significant associations between medications/serum biomarkers and anthropometric parameters [*z*-scores based on chronological age (*n* = 82)] - unadjusted analyses

Variable	Mid-arm circumference CA-*z*-scores	Subscapular skinfold CA-*z*-scores	Weight CA-*z*-scores	BMI CA-*z*-scores
Adalimumab		0.90[1] (0.08, 1.72)[2] 0.033[3]		1.09 (0.05, 2.13) 0.04
Azathioprine	-0.65 (-1.25, -0.05) 0.033	-0.5 (-0.85, -0.14) 0.006	-0.7 (-1.16, -0.23) 0.004	-0.56 (-1.004, -0.12) 0.014
Methotrexate		0.75 (0.06, 1.43) 0.032		
ESR	-0.024 (-0.05, -0.002) 0.036		-0.03 (-0.05, -0.02) 0.0003	-0.02 (-0.04, -0.006) 0.009
Hemoglobin			0.19 (0.04, 0.34) 0.015	

[1]Regression coefficient for unadjusted analyses [*i.e.*, one medication or serum biomarker (independent variable) per model and no adjustment for potential confounding]; [2](95% confidence interval); [3]*P*-value. BMI: Body mass index; CA *z*-score: *z* score based on chronological age; ESR: Erythrocyte sedimentation rate.

azathioprine/6-mercaptopurine and BA *z*-scores for these specific anthropometric parameters, the direction of the association remained negative between thiopurines and subscapular skinfold BA *z*-scores (though did not achieve statistical significance due to smaller sample size (*n* = 49 for BA analyses *vs n* = 82 for CA analyses). This continued negative association between thiopurines and subscapular skinfold BA *z*-scores in combination with our previously reported finding of a negative association between thiopurines and standardized BA results[11] calls into question the efficacy of thiopurines for treating pediatric CD. Our findings highlight the importance of considering BA in the interpretation of anthropometric parameters because its inclusion clarifies the relationship between medications and these outcomes.

Previously published data on the impact of thiopurines on anthropometry for comparison to our findings are limited, but also raise concerns about the efficacy of these medications. Csontos *et al*[31] reported no statistically significant difference in the change in fat free mass index, skeletal muscle index, or body fat mass index in adult IBD patients on *vs* not on azathioprine during initiation of biologic therapy. In newly diagnosed CD children randomized to treatment with 6-mercaptopurine plus steroids *vs* placebo plus steroids, Markowitz *et al*[46] did not detect a difference in statural growth.

Regarding a possible negative impact of utilizing thiopurines, in a pediatric IBD cohort, Hyams *et al*[47] reported thiopurine exposure is an important preceding event for the development of malignancy or hemophagocytic lymphohistiocytosis. Our data identify another negative signal associated with thiopurines, given the constellation of findings of statistically significant negative associations between azathioprine/6-mercaptopurine and mid-arm circumference, subscapular skinfold, weight and BMI CA *z*-scores and persistent negative association with subscapular skinfold BA *z*-scores (though did not achieve statistical significance due to smaller sample size available for BA analyses), in combination with our previously reported finding of a statistically significant association with lower standardized BA results[11]. Prospective longitudinal study is required to examine the longitudinal pattern of these associations and to investigate whether

Table 4 Significant associations between medications/serum biomarkers and anthropometric parameters [*z*-scores based on chronological age (*n* = 82)] - adjusted analyses

Variable	Mid-arm circumference CA-*z*-scores	Subscapular skinfold CA-*z*-scores	Weight CA-*z*-scores	BMI CA-*z*-scores
Adalimumab		1.02[1] (0.18, 1.86)[2] 0.018[3]		1.17 (0.13, 2.21) 0.029
Azathioprine	-0.64 (-1.26, -0.02) 0.045	-0.47 (-0.83, -0.10) 0.014	-0.73 (-1.17, -0.29) 0.002	-0.58 (-1.03, -0.12) 0.013
Methotrexate		0.81 (0.10, 1.53) 0.027		
ESR			-0.03 (-0.05, -0.01) 0.01	-0.03 (-0.05, -0.004) 0.024

[1]Regression coefficient for adjusted analyses [*i.e.*, one medication or serum biomarker (independent variable) per model and CA at study visit, sex, CRP, albumin, ESR and hemoglobin are included in the model to adjust for potential confounding]; [2](95% confidence interval); [3]*P*-value. BMI: Body mass index; CA *z*-score: *z* score based on chronological age; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

Table 5 Significant associations between serum biomarkers and anthropometric parameters [*z*-scores based on bone age (*n* = 49)] - unadjusted analyses

Variable	Mid-arm circumference BA-*z*-scores	Subscapular skinfold BA-*z*-scores	Triceps skinfold BA-*z*-scores	Weight BA-*z*-scores	BMI BA-*z*-scores
WBC	-0.19[1] (-0.36, -0.02)[2] 0.029[3]	-0.12 (-0.24, -0.01) 0.04		-0.16 (-0.28, -0.04) 0.008	
ESR				-0.03 (-0.04, -0.01) 0.003	-0.02 (-0.03, -0.001) 0.037
CRP			-0.03 (-0.07, -0.0001) 0.049	-0.06 (-0.10, -0.02) 0.008	
Albumin				0.73 (0.28, 1.18) 0.002	
IGF-1 BA-*z*-scores				0.2 (0.01, 0.38) 0.039	

[1]Regression Coefficient for Unadjusted Analyses [*i.e.*, one serum biomarker (independent variable) per model and no adjustment for potential confounding]; [2](95% confidence interval); [3]*P*-value. BA *z*-score: *z* score based on bone age; BMI: Body mass index; WBC: White blood cell; ESR: Erythrocyte sedimentation rate; IGF-1: Insulin-like growth factor-1; CRP: C-reactive protein.

these findings represents a lack of efficacy of thiopurines (given that anthropometric parameters and skeletal maturation reflect nutritional status/disease status) *vs* a direct negative impact of thiopurines in pediatric CD. Patients with lower body composition *z*-scores and lower standardized BA results were not selectively placed on thiopurines *vs* another medication such as methotrexate, infliximab, or adalimumab as these measurements were obtained at the time of the study.

Adalimumab and methotrexate were positively associated (statistically significant) with measurements of fat mass [subscapular CA *z*-scores (adalimumab/methotrexate)] and BMI CA *z*-scores (adalimumab). While these medications were not statistically significantly associated with these outcome BA *z*-scores due to a smaller sample size available for BA analyses, the direction of these associations (positive) remained unchanged and the effect sizes were similar to only mildly decreased compared with the statistically significant positive associations between these medications and these outcome CA *z*-scores, supporting a positive association between adalimumab and methotrexate with these anthropometric parameter BA *z*-scores.

Similar to our finding of a positive association between the anti-tumor necrosis factor alpha (TNF-α) agent, adalimumab, and BMI, Diamanti *et al*[48] reported that weight and BMI improved in children treated with infliximab, but not with mesalazine and azathioprine. Wiese *et al*[49] reported a significant increase in BMI with infliximab treatment in adult CD.

In a pediatric CD study, investigators reported specific medications were associated with greater increases in race- and sex-specific *z*-scores for both lean mass (infliximab) and fat mass (infliximab, glucocorticoid,

Table 6 Significant associations between medications/serum biomarkers and height *z*-scores by sex (based on chronological age (female *n* = 35; male *n* = 47)) - unadjusted analyses

Variable	Height CA *z*-scores	Point estimate[1]	95%CI	*P* value
Infliximab	Females	0.65	0.04, 1.25	0.038
CRP	Males	-0.04	-0.079, -0.002	0.039
ESR	Males	-0.03	-0.051, -0.011	0.003
Hemoglobin	Males	0.23	0.04, 0.42	0.018
Platelets	Males	-0.004	-0.006, -0.001	0.005

[1]Regression coefficient for unadjusted analyses [*i.e.*, one medication or serum biomarker (independent variable) per model and no adjustment for potential confounding]. CA *z*-score: *z* score based on chronological age; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

Table 7 Significant associations between serum biomarkers and height *z*-scores [based on bone age (*n* = 49)] - unadjusted analyses

Variable	Height BA-*z*-scores	Point estimate[1]	95%CI	*P* value
Albumin	Males and females	0.8	0.22, 1.36	0.008
CRP	Males and females	-0.06	-0.11, -0.006	0.03
ESR	Males and females	-0.02	-0.05, -0.003	0.029
IGF-1 BA-*z*-scores	Males and females	0.26	0.03, 0.48	0.025

[1]Regression coefficient for unadjusted analyses [*i.e.*, one serum biomarker (independent variable) per model and no adjustment for potential confounding]. BA *z*-score: *z* score based on bone age; BMI: Body mass index; ESR: Erythrocyte sedimentation rate; IGF-1: Insulin-like growth factor-1; CRP: C-reactive protein.

and methotrexate) relative to height[50]. Similarly, we identified a positive association between methotrexate and subscapular skinfold CA *z*-scores. In a CD patient cohort, age 5-25 years, Sentongo *et al*[14] reported triceps skinfold *z*-scores, also a measure of adiposity, were significantly correlated with corticosteroid exposure. Our findings do not reveal a statistically significant association between history of corticosteroid therapy and current anthropometric parameters.

Csontos *et al*[31] reported baseline BMI increased significantly during initiation of adalimumab/infliximab therapy in adult IBD, in agreement with our identified positive association between adalimumab and BMI. They found fat free mass index also increased. They found no significant differences between the effects of adalimumab and infliximab on body composition, whereas we identified significant associations between body composition and adalimumab only, not infliximab. Notably, fat free mass index and skeletal muscle mass index significantly improved only in males. Subramaniam *et al*[51] reported infliximab was associated with significant gains in muscle volume that correlated with male sex in adult CD[51]. Supporting these sex differences in response to infliximab, in a mouse model of pulmonary inflammation in which TNF-α was over expressed in mouse lungs, lower body and muscle mass were evident only in males[52].

Our study does not reveal a sex difference in the association between medications and body composition, but does identify a statistically significant positive association between infliximab and height CA *z*-scores in females only. A positive relationship between infliximab and height BA *z*-scores was also identified in females only, but did not reach statistical significance, likely due to the smaller sample size available for BA analyses (*n* = 17 females for BA analyses *vs n* = 35 females for CA analyses). The combination of findings described here between infliximab and height *z*-scores (based on CA and BA) supports a possible sex difference in response to infliximab from the standpoint of statural growth. Taken together, these findings of sex differences in response to infliximab add to the growing body of literature indicating that there may be sex differences in the molecular pathways affecting statural growth and body composition in CD. Our findings in combination with the existing literature raise an intriguing question: does TNF-α play an important role in compromising body composition in CD males but statural growth in females, and if so, why? Tang *et al*[52] speculated that estrogen has protective effects against the actions of TNF-α. Ordas *et al*[53] reported that clearance of monoclonal antibodies is higher in men. Ternant *et al*[54] theorized that the central volume of distribution may be higher in men because for a given body weight, plasma volume is lower in women.

We found hemoglobin was positively associated, while platelets, ESR, and CRP were negatively associated, with height CA *z*-scores in males only, supporting our previously reported findings of a greater detrimental effect of inflammation on statural growth in males[10]. Several investigators have documented that growth impairment is more frequent in males[9,10,14,21,32-35]. Perhaps the molecular pathways that lead to growth impairment in males are different than in females, and less responsive to currently used medications, such as infliximab. As expected, albumin and IGF-1 BA *z*-scores were positively associated, while ESR and CRP were negatively associated with height BA *z*-scores. In contrast, no treatment (5-aminosalicylate, corticosteroids, immunomodulators, infliximab, nutritional therapy,

surgical resection) was associated with height, weight or BMI at maximal follow up in a pediatric CD cohort in Northern France[21].

The relationships between medications and anthropometric parameters may reflect efficacy of medications, side effects of medications, or confounding by indication. Since body composition measurements were obtained as part of a study protocol and not standard of care, it is unlikely these relationships reflect confounding by indication since these body composition measurements were not available to the care provider. Our results suggest methotrexate, infliximab and adalimumab are more effective than thiopurines for treating pediatric CD.

As expected, body composition BA *z*-scores were systematically higher than corresponding body composition CA *z*-scores. Patients did not exhibit severe deficiencies in fat stores, as reflected by standardized subscapular and triceps skinfold measurements. Depending on the measurement obtained, 3% to 11% had subscapular or triceps skinfold measurement CA *z*-scores or BA *z*-scores > 2.0, reflecting excess fat stores. In contrast, 16%-17% had deficiencies in lean mass tissue as reflected by mid-arm circumference *z*-score measurements < -2.0 and only 2% with mid-arm circumference *z*-score measurements > 2.0. We identified a negative association between thiopurines and mid-arm circumference CA *z*-scores. The published literature surrounding the relationship between medications and lean mass tissue is conflicting[31,50-51]. More studies are needed to identify the most effective treatments for improving lean mass tissue in pediatric CD.

Correlations between inflammatory markers/disease activity indices and anthropometric parameters have been reported by other investigators[5,14,50,55,56], similar to our findings. Enhancing our understanding of the specific inflammatory cytokines involved in molecular pathways affecting body composition and growth is critical for optimizing treatment.

Limitations

The etiology of compromised nutritional status/disease status is multifactorial. The cross-sectional study design does not permit longitudinal assessment of changes in anthropometric parameters with respect to medication treatment and serum biomarkers to be determined. Within-subjects characterization of the influence of disease activity and hormone levels on changes in anthropometric parameters may clarify the effects of long-term inflammation on nutritional status/disease status. Nevertheless, our results suggest a mechanistic relationship between medications, inflammation and anthropometric status/disease status, as well as a difference by sex. Prospective longitudinal study, collecting additional markers of disease activity/disease status such as fecal calprotectin, cross-sectional imaging and endoscopic assessment, is required as a next step to further investigate these intriguing findings and would allow further risk stratification which will improve patient counseling, guide expectations, and facilitate an individualized treatment approach. Future studies should examine the impact of monotherapy *vs* combination therapy (including duration of treatment and drug levels) on anthropometric status/disease status.

Summary and Conclusions

Complex processes regulate body composition and growth in pediatric CD. We examined the relationship between medication treatments and serum inflammatory and hormonal biomarkers with anthropometric parameters in a well-characterized pediatric CD cohort. Our findings reinforce the importance of accounting for BA when interpreting anthropometric parameters in pediatric CD. The main findings of our study raise intriguing questions.

Thiopurines were negatively associated with specific anthropometric parameters. Do thiopurines have a negative effect on nutritional status/disease status? Alternatively, is the efficacy of thiopurines suboptimal? This interesting finding may have significant implications for pediatric CD treatment and requires further investigation in a prospective longitudinal study to determine if thiopurines should continue to be utilized as a treatment for pediatric CD.

Infliximab was positively associated with standardized height in females only. Is there a sex difference in response to infliximab from the standpoint of statural growth? Specific serum biomarkers were associated with standardized height in males only, supporting the hypothesis that inflammation has a more detrimental effect on statural growth in males. The combination of these findings lends further support to the theory that sex differences in the molecular pathways driving statural growth impairment in pediatric CD exist and should be delineated in a prospective longitudinal study utilizing height velocity BA *z*-scores as the primary outcome. An improved understanding of this sex difference in response to treatment would be a huge step towards enhancing risk prediction and individualized treatment.

The studies presented herein contribute to a better understanding of the relationship between medications and serum inflammatory and hormonal biomarkers with anthropometric parameters in pediatric CD. These findings serve as a foundation on which to build future studies with the goal of identifying patients at highest risk for poor outcomes, enhancing treatment algorithms, and ultimately developing individual treatment approaches based on risk stratification. The present study may provide a basis for mechanistic studies in many pediatric chronic inflammatory conditions.

ARTICLE HIGHLIGHTS

Research background

Similar to impaired statural growth (height velocity), a dynamic marker of

disease status, body composition deficits may reflect poorly controlled disease despite the absence of overt clinical intestinal symptoms. Delayed bone age (BA) is common in pediatric Crohn's disease (CD). Determination of BA allows clinically meaningful interpretation of growth in the context of skeletal maturity in pediatric CD. The impact of accounting for BA in the interpretation of body composition is unclear. Accurate interpretation of body composition is important since it reflects nutritional and disease status. Not only is nutritional status an important determinant of pubertal development and growth velocity, it is a prognostic factor for disease course. The association between medications and serum inflammatory and hormonal biomarkers with anthropometric measurements is not well delineated in pediatric CD, particularly after adjusting for maturational status (BA).

Research motivation

Nutritional status is an important factor to consider when making therapeutic decisions given its association with poor outcomes. Yet, the impact of treatments on anthropometric measurements is poorly defined and has not received sufficient attention.

Research objectives

Our aims were to determine the distribution of anthropometric parameters based on CA (CA *z*-scores) and BA (BA *z*-scores) and characterize the associations between medications and serum biomarkers with anthropometric parameter *z*-scores in pediatric CD.

Research methods

CD patients [< chronological age (CA) 21 years] were prospectively enrolled in a cross-sectional study. Descriptive statistics were generated for participants' demographic characteristics and key variables of interest. Paired *t*-tests were used to compare anthropometric parameter *z*-scores calculated based on CA (CA *z*-scores) and BA (BA *z*-scores) for interpretation of anthropometric parameters. Linear regression was utilized to examine associations between medications and serum biomarkers with anthropometric parameter *z*-scores calculated based on CA (n = 82) and BA (n = 49). We reported regression coefficients as well as their corresponding p-values and 95% confidence intervals.

Research results

Mean CA at the time of the study visit was 15.3 ± 3.5 (standard deviation; range = 4.8-20.7) years. Mean triceps skinfold, subscapular skinfold and mid-arm circumference (MAC) BA *z*-scores were higher than corresponding CA *z*-scores. Medications were positively associated with subscapular skinfold (adalimumab and methotrexate) and BMI (adalimumab) CA z-scores. Azathioprine/6-mercaptopurine were negatively associated with MAC, subscapular skinfold, weight and BMI CA *z*-scores . ESR, CRP, and WBC count were negatively associated, while albumin and IGF-1 BA *z*-scores were positively associated with specific AP *z*-scores. Mean height CA *z*-scores were higher in females, not males, treated with infliximab. Hemoglobin was positively associated, while platelets, ESR and CRP were negatively associated with height CA *z*-scores in males, not females.

Research conclusions

Our findings reinforce the importance of accounting for BA when interpreting anthropometric parameters in pediatric CD. The main findings of our study raise intriguing questions. Thiopurines were negatively associated with specific anthropometric parameters. Do thiopurines have a negative effect on nutritional status/disease status? Alternatively, is the efficacy of thiopurines suboptimal? Infliximab was positively associated with standardized height in females only. Is there a sex difference in response to infliximab from the standpoint of statural growth? Specific serum biomarkers were associated with standardized height in males only, supporting the hypothesis that inflammation has a more detrimental effect on statural growth in males. Our results suggest a mechanistic relationship between medications, inflammation and anthropometric status/disease status, as well as a difference by sex. The studies presented herein contribute to a better understanding of the relationship between medications and serum inflammatory and hormonal biomarkers with anthropometric parameters in pediatric CD. Prospective longitudinal study is required as a next step to further investigate these intriguing findings and would allow further risk stratification which will improve patient counseling, guide expectations, and facilitate an individualized treatment approach.

Research perspectives

These findings serve as a foundation on which to build future studies with the goal of identifying patients at highest risk for poor outcomes, enhancing treatment algorithms, and ultimately developing individual treatment approaches based on risk stratification. The present study may provide a basis for mechanistic studies in many pediatric chronic inflammatory conditions.

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