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Editorial Board Member of *World Journal of Gastroenterology*, Ragesh Babu Thandassery, MD, Assistant Professor, Doctor, Department of Medicine, Central Arkansas Veterans Healthcare Systen, Little Rock, AR 72205, United States. doc.ragesh@gmail.com

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REVIEW

Predictors of early and late hepatocellular carcinoma recurrence

Riccardo Nevola, Rachele Ruocco, Livio Criscuolo, Angela Villani, Maria Alfano, Domenico Beccia, Simona Imbriani, Ernesto Claar, Domenico Cozzolino, Ferdinando Carlo Sasso, Aldo Marrone, Luigi Elio Adinolfi, Luca Rinaldi

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Riccardo Nevola, Rachele Ruocco, Livio Criscuolo, Angela Villani, Maria Alfano, Domenico Beccia, Simona Imbriani, Domenico Cozzolino, Ferdinando Carlo Sasso, Aldo Marrone, Luigi Elio Adinolfi, Luca Rinaldi, Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli," Naples 80138, Italy

Riccardo Nevola, Ernesto Claar, Internal Medicine and Hepatology Unit, Ospedale Evangelico Betania, Naples 80147, Italy

Corresponding author: Riccardo Nevola, MD, PhD, Doctor, Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli," Piazza Miraglia, Naples 80138, Italy. riccardo.nevola@unicampania.it

Abstract

Hepatocellular carcinoma (HCC) is the most frequent liver neoplasm, and its incidence rates are constantly increasing. Despite the availability of potentially curative treatments (liver transplantation, surgical resection, thermal ablation), long-term outcomes are affected by a high recurrence rate (up to 70% of cases 5 years after treatment). HCC recurrence within 2 years of treatment is defined as "early" and is generally caused by the occult intrahepatic spread of the primary neoplasm and related to the tumor burden. A recurrence that occurs after 2 years of treatment is defined as "late" and is related to de novo HCC, independent of the primary neoplasm. Early HCC recurrence has a significantly poorer prognosis and outcome than late recurrence. Different pathogenesis corresponds to different predictors of the risk of early or late recurrence. An adequate knowledge of predictive factors and recurrence risk stratification guides the therapeutic strategy and post-treatment surveillance. Patients at high risk of HCC recurrence should be referred to treatments with the lowest recurrence rate and when standardized to combined or adjuvant therapy regimens. This review aimed to expose the recurrence predictors and examine the differences between predictors of early and late recurrence.

Key Words: Hepatocellular carcinoma; Early recurrence; Late recurrence; Predictors; Liver transplant; Liver resection; Thermal ablation

Core Tip: Hepatocellular carcinoma is burdened by a high rate of both early and late recurrence. The knowledge of the predictive factors of recurrence and its risk stratification should allow optimization of the management of the patient with hepatocellular carcinoma.

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INTRODUCTION

Liver cancers represent the fifth neoplasm by incidence and the fourth cause of cancer-related deaths worldwide, with a higher incidence in men than in women[1] and an epidemiological distribution that results from the variations of etiological factors for liver disease (with a current reduction in viral hepatitis and an increase in metabolic etiologies)[2,3]. Hepatocellular carcinoma (HCC) accounts for about 90% of liver cancer cases, with an increasing incidence of 75% from 1990 to 2015[1,4]. The onset of HCC generally follows chronic liver damage resulting in the development of fibrosis and especially liver cirrhosis, although cases of HCC in the absence of liver damage are possible and are generally related to aflatoxin exposure[5].

The main risk factors for HCC are represented by: liver cirrhosis and chronic hepatitis; chronic hepatitis B virus (HBV) infection (with or without hepatitis D virus)[6,7] or hepatitis C virus (HCV)[8,9]; chronic alcohol abuse; metabolic syndrome[10]; diabetes mellitus[11]; and obesity[12]. In particular, 1% to 8% of patients with liver cirrhosis develop HCC annually[13]. Low platelet count, severe portal hypertension, certain comorbidities (*e.g.*, obesity, diabetes mellitus), cigarette smoking, concomitant alcohol consumption, older age and male sex are factors closely associated with the development of HCC in patients with liver cirrhosis[13-17].

The mortality rate of HCC patients, although improving, still appears to be extraordinarily high[18]. An early diagnosis and an appropriate therapeutic approach contribute to higher overall and disease-free survival rates. In particular, when diagnosed at an early stage, HCC can be effectively treated by liver transplantation (LT) or loco-regional techniques, including liver resection (LR) and radio-frequency ablation (RFA). Optimal treatment choice is based on both tumor burden and residual liver function[19-21]. HCC recurrence rates after LT accounts for about 13% of cases[22], and these rates appear significantly higher after loco-regional treatment, reaching 70% of cases at 5 years[4]. The recurrence of the neoplasm may reflect both the presence of intrahepatic metastases ("true" or "early" recurrence) and the development of de novo tumors ("late" recurrence). A 2-year cutoff is generally used to define the two entities[4].

There are numerous factors (related to the patient, the tumor and the type of treatment) able to predict the risk of recurrence after loco-regional treatment or LT. Although many predictors are common to early and late recurrence, other factors may be exclusive to one form due to the different pathogenesis. An adequate recurrence risk stratification would allow personalization of the therapeutic strategy for each patient, optimization of the surveillance program and ideally encourage the study, validation and use of adjuvant therapy in high-risk patients.

The aim of this review was to examine the predictors of HCC recurrence after LT and loco-regional treatment and discuss the differences between predictors of early and late recurrence.

STRATEGIES OF HCC RADICAL TREATMENT

In order to define the most appropriate therapeutic strategy able to maximize outcomes and reduce the risk of recurrence, HCC requires appropriate staging that considers cancer-related factors and residual liver function. The Barcelona Clinic Liver Cancer (BCLC) staging system is the most used to predict patient prognosis and establish treatment allocation[4]. It divides patients into 5 categories (0, A-D) based on tumor characteristics (uni- or multifocality, vascular invasion, size and extrahepatic spread) and liver function (assessed by Child Pugh score).

"Very early HCC stage" (BCLC 0) is defined as patients with preserved liver function and carcinoma in situ (single HCC lesion < 2 cm without vascular invasion), whereas "early stage" (BCLC A) is defined as patients with preserved liver function and a single lesion > 2 cm or 3 lesions with diameters < 3 cm. Patients at BCLC stage 0 and A are optimal candidates for a radical therapeutic strategy (LT, ablation, surgical resection). Patients in more advanced states (BCLC B-D) are candidates for palliative (chemoembolization, systemic therapy) or supportive treatment[4,21].

Due to the excellent long-term outcomes achievable, LT is considered the gold standard for the treatment of HCC[22,23]. It is indicated as the first-line therapy in patients with HCC who meet the Milan criteria (single tumor ≤ 5 cm or multiple tumors as ≤ 3 nodules size ≤ 3 cm) without vascular invasion and/or extrahepatic involvement[24]) but are not eligible for LR[4]. Recent evidence also suggests that patients beyond the Milan criteria could be reconsidered for LT after adequate downstaging of the tumor[25]. LR is the treatment of choice in HCC patients with or without cirrhosis with well-preserved liver function, solitary tumors and clinically mild portal hypertension (hepatic vein to portal system gradient ≤ 10 mmHg)[4,26], with the possibility (not standardized yet) to extend the indication to multicentric tumors or to tumors > 2 cm[4]. It is still one of the main local-regional curative-intent treatments, with a 5-year survival between 40% and 70%[27].

Thermal ablation with RFA or microwaves is the standard of care for HCC patients with BCLC 0 and A stage not suitable for surgery (LT or LR) or as an alternative to LR in very early HCC stage with favorable localization also in patients eligible for surgery[4]. Thermoablation is also indicated as a neoadjuvant therapy in patients who are candidates for LT (pre-transplant) in order to reduce the risk of recurrence. Selected patients with intermediate HCC-BCLC B (solitary > 3 cm or > 3 nodules < 3 cm or advanced liver failure not clinically decompensated) can be reasonably treated with RFA, even if medium- and long-term outcomes appear worse than patients with HCC BCLC 0 or A[28].

Transarterial chemoembolization (TACE) is the most widely used treatment for unresectable HCC and offers significant overall survival advantages over best supportive care[4], although it has no clear curative purposes and cannot be considered a radical approach. In particular, the survival rates after TACE are 70.3% at 1 year, 51.8% at 2 years, 40.4% at 3 years and 32.4% at 5 years[29]. The rationale of the technique is based on the intermediate HCC intense arterial neo-angiogenic activity and consists in the intra-arterial administration of cytotoxic agents (doxorubicin or platinum derivatives) followed by the embolization of peritumoral vessels inducing a cytotoxic and ischemic effect of the tumor mass. Combination treatments (*e.g.*, TACE + RFA) and neoadjuvant or adjuvant systemic treatments are currently being studied.

EARLY OR LATE RECURRENCE

The recurrence of HCC represents a relevant clinical issue, affecting up to 70% of patients undergoing curative treatment[4]. Although many factors are involved in the decision-making process of the best possible treatment (Figure 1), the assessment of baseline risk recurrence significantly influences the choice of treatment type and its timing. Moreover, the treatment of a relapse is generally more complex than the treatment of the first lesion, due to the change in anatomical or functional liver conditions and a more advanced age. Patients at high risk of early recurrence are candidates for close surveillance and potentially adjuvant therapy, which has not yet been standardized[30,31].

The "true" recurrence (secondary to the presence of occult intrahepatic metastases) is generally "early" and accounts for more than 70% of tumor recurrence. It derives from an intrahepatic dissemination of the neoplasm through the portal circulation. A 2-year cutoff is used to distinguish it from late recurrence, generally resulting from de novo development of the neoplasm. However, this cutoff remains arbitrary and not universally accepted. Yamamoto *et al*[32], for example, identified 17 mo as the best cutoff to distinguish early and late recurrence on a cohort of 252 patients with recurrence of HCC after hepatectomy. Previously Hayashi *et al*[33] had hypothesized a 1 year cutoff instead. A further shorter observation period has recently been hypothesized by Xing *et al*[34] who identify 8 mo as the ideal threshold to define a recurrence of HCC as early or late. Although the temporal criterion is used to distinguish a pathogenesis related to the presence of occult intrahepatic metastases or to a de novo neoplasm, a precise differentiation would require the study of recurrence clonality by genetic/genomic analyzes[35].

The two types of recurrence are two distinct entities associated with different risk factors. Early recurrence is associated with tumor-related factors, whereas late recurrence is related to underlying liver disease. They have a substantially different clinical and biological profile and are burdened by different morbidity and mortality. In fact, an early recurrence represents a significant negative prognostic factor and has a far greater impact on overall survival than a late recurrence[32,36]. Xing *et al*[34] demonstrated that patients with early recurrence showed a median of survival free from cancer of 8.4 mo (7.5-10.0 mo) compared to a median of 21.3 mo (17.9-23.8 mo) for patients with late recurrence. In particular, an early recurrence, mainly determined by the aggressiveness of the primary tumor, is characterized by larger dimensions, higher rates of multifocality and intrahepatic spread, higher probability of vascular invasion and higher levels of alpha-fetoprotein (AFP) compared to a recurrence that develops later[36]. Conversely, late recurrence is generally related to etiology and cirrhosis, risk factors for hepatocarcinogenesis, and not to the primary tumor[37].

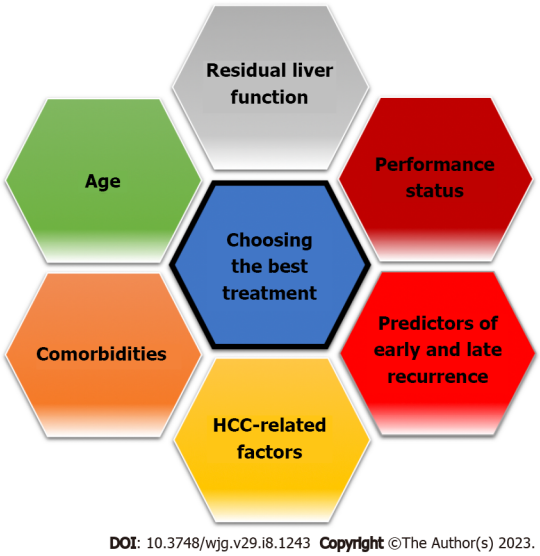

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Figure 1 Determinants of therapeutic decision making for hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

EARLY RECURRENCE PREDICTIVE FACTORS

The factors affecting the possibility of early HCC relapse after LT or loco-regional treatment can be classified into three categories: those related to the tumor (size, number of nodules, differentiation, oncological markers); to the patient (*e.g.*, age, comorbidity, liver function, possible viral load, presence and activity of hepatitis, presence and activity of liver cirrhosis); and treatment (type of treatment, margins, characteristics of resection) (Table 1)[38,39].

Predictors of early recurrence related to HCC characteristics

Several factors related to the characteristics of HCC are able to predict the risk of early recurrence (Table 1). This risk is closely related to the tumor burden and its aggressiveness, which increases the probability of occult intrahepatic spread and affects the radicality of the treatment[40].

Preoperative radiological imaging can already provide first indications about the risk of relapse. In this regard, the size of the tumor represents a relevant predictor of the risk of early recurrence. Jung *et al*[36] showed that a maximum diameter of HCC greater than 3 cm before a curative LR represented an independent risk factor for early recurrence (defined by the authors as occurring within 1 year of treatment). These results have recently been confirmed by Lee *et al*[41]. Zhu *et al*[42] identified a 4.77-fold higher risk of early recurrence after an LR for cancers with a maximum diameter greater than 2.6 cm compared to those with a smaller maximum diameter[42]. Similarly, the multifocality of HCC seems to be another significant risk factor for early recurrence[41]. Tumor size and number of lesions represents together the aforementioned Milan criteria. As expected, being beyond the Milan criteria represents an independent risk factor for early HCC recurrence[43].

At histological evaluation, the most predictive factors of the risk of early HCC recurrence are the presence of microvascular invasion, the integrity of the capsule and the degree of differentiation. In light of the fact that early recurrence is generally due to intrahepatic dissemination of the neoplasm through the portal circulation, it is not surprising that vascular invasion has been identified many times as an independent predictor of recurrence. If the involvement of the major vessels (*e.g.*, portal vein, inferior vena cava) can be evaluated radiologically and often excludes the possibility of loco-regional treatment for radical purposes, microvascular invasion needs to be evaluated by histopathological analysis of the intraoperative sample. Microvascular invasion, although dependent upon the operator and influenced by possible sampling errors, represents an independent risk factor for early recurrence[34,44,45] affecting approximately one-third of HCC cases that underwent LR[37]. Since a qualitative and/or quantitative classification of microvascular invasion is not yet available, its only presence/absence is generally considered in the histopathological evaluation. However, in this regard, Roayaie *et al*[46] proposed a risk system based on histological features of microvascular invasion that includes invasion of a vessel with a muscular wall and invasion of vessels ≥ 1 cm away from the tumor capsule. According to the authors, this system stratifies patients into three distinct groups with significantly different risks of recurrence and death. In particular, patients with microvascular invasion and the aforementioned risk factors show outcomes (tumor recurrence rate, mortality rate) comparable to patients with macroscopic vascular invasion.

The integrity of the HCC capsule would instead represent a protective factor for the risk of recurrence [42]. The capsule would act as a barrier to the spread and metastasis of cancer cells. This function is lost

Table 1 Predictors of early hepatocellular carcinoma recurrence after curative treatment

Predictors related to HCC characteristics	Ref.	Predictors related to patient characteristics	Ref.	Predictors related to treatment	Ref.
Size		Liver cirrhosis	[37,41]	RFA (*vs* LR)	[41,88,89]
> 2.6 cm	[42]				
> 3 cm	[36,41]				
Multifocality	[41]	Liver insufficiency (CPS, MELD, ALBI)	[37,56-58]	LR:	
				Liver resection margins invasion	[44]
				Liver resection margins < 1 cm	[43,86]
				Non-anatomical resection[1]	[45]
Beyond Milan criteria	[43]	High total bilirubin	[42]	RFA: ablation margins < 1 cm	[91]
Microvascular invasion	[34,36,38,40,44,45]	Male sex	[37]		
Lack of capsule integrity	[34,42]	High PLR	[49,59,60]		
Poor histological differentiation	[36,43,47]	High NLR	[49,60]		
High AFP	[36,41,50]	DAAs therapy			
> 10 ng/mL	[48]	Predictor	[62,63]		
> 32 ng/mL	[45]	Non-predictor	[64-76]		
> 400 ng/mL	[40]	Protective	[77-83]		
High PIVKA-II	[41,52,55]	High viral load in HBV infected patients	[109]		
> 46.0 mAU/mL	[38]				
> 375.5 mAU/mL	[51]				

[1]Available data are contradictory and not conclusive.

AFP: Alpha-fetoprotein; ALBI: Albumin-Bilirubin score; CPS: Child-Pugh score; DAA: Direct antiviral agent; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LR: Liver resection; MELD: Model for end-stage liver disease; NLR: Neutrophil-to-lymphocyte ratio; PIVKA-II: Protein induced by vitamin K absence or antagonist II; PLR: Platelet-to-lymphocyte ratio; RFA: Radio-frequency ablation.

in non-capsular HCC or those with a ruptured capsule[34,42]. Zhu *et al*[42] showed that non-capsular HCC was associated with a higher rate of poorer differentiation and vascular invasion than capsular HCC.

Histological differentiation is considered an independent risk factor for early HCC recurrence. It is classified according to the Edmonson-Steiner criteria: 1st and 2nd degree correspond to well differentiated neoplasms; and 3rd and 4th degree correspond to poorly differentiated neoplasms. In particular, grades 3 and 4 are related to a significantly increased risk of early recurrence[36,43]. In patients without microvascular invasion, the degree of differentiation seems to be the best histological predictor of the risk of early recurrence and overall survival[47].

In light of the growing evidence of the efficacy in predicting the risk of recurrence, histological evaluation will become crucial in choosing the optimal therapeutic strategy for each patient, allowing the implementation of the stratification of the basal recurrence risk with the postoperative one. Patients at high risk of recurrence should undergo an aggressive surveillance strategy and ideally adjuvant therapy.

Among the predictors of early recurrence, the markers expressed by the neoplasm are certainly the most studied. In particular, AFP, a glycoprotein physiologically produced by the liver and fetus, can increase in pathological conditions, such as liver cancer. In addition to the diagnostic phase, it plays an important role as a predictor and monitoring tool in the context of HCC recurrence. There is almost unanimous agreement on the predictive value of elevated AFP levels for the risk of early HCC recurrence after treatment[36,48,49], but there are no standardized cutoffs pre- and post-treatment.

Jung *et al*[36] showed that high AFP values both pre- and post-hepatectomy were associated with a higher risk of early recurrence (which the authors defined as < 1 year after treatment) but without a standardized threshold value. Using a retrospective data analysis, Kim *et al*[48] showed that preoperative AFP values > 10 ng/mL were a predisposing factor of disseminated HCC recurrence

within 3 mo after hepatectomy for solitary HCC [odds ratio: 5.333; 95% confidence interval (CI): 1.095–25.985]. Fang *et al*[40] showed instead that preoperative AFP > 400 ng/mL correlated independently with the risk of early recurrence. Recently, AFP levels at 12 wk after achieving sustained virological response with direct antiviral agents (DAAs) treatment in chronic HCV patients have also been independently associated with a risk of HCC recurrence[50].

The protein induced by vitamin K absence or antagonist II (PIVKA-II) is an immature form of prothrombin, a cofactor of vitamin K that is synthesized by the liver when the latter is not produced or antagonized. Evaluation of PIVKA-II has been shown to be useful in diagnosing HCC and in stratifying the risk of recurrence[38,41,51,52]. In fact, it acts as a growth factor, promoting cell proliferation and tumor angiogenesis in HCC patients[52,53]. The evaluation of PIVKA-II appears to be complementary to AFP[54]. Low values of both AFP and PIVKA-II are associated with a better prognosis compared to elevated levels of AFP and/or PIVKA-II[55]. Serial measurements of both proteins allow a prompt diagnosis of early recurrence. In fact, pre- and post-treatment levels of PIVKA-II are good predictors of early HCC recurrence[38,41,51] since they are related to greater aggressiveness of the neoplasm, such as the presence of vascular invasion and intrahepatic metastasis of HCC cells[52].

Similarly to what is highlighted for AFP, there is no agreement on a PIVKA-II cutoff that is useful as a guide for assessing the risk of recurrence. In this regard, Wang *et al*[51] showed that in patients with a low risk of recurrence (tumor size < 5 cm, single tumor, absence of satellite lesions, absence of vascular invasions, high degree of histological differentiation, BCLC stage 0-A), a preoperative value of PIVKA-II > 375.5 mAU/mL was the strongest independent prognostic factor for the risk of early recurrence [hazard ratio (HR): 2877; 95%CI: 1524-5429]. Furthermore, patients who expressed high levels of PIVKA-II showed lower 1-year time-to-progression than patients with low levels (54.8% *vs* 20.2%, respectively). More recently, Hong *et al*[38] identified a cutoff of 46 mAU/mL for PIVKA-II for predicting an increased risk of early recurrence.

Predictors of early recurrence related to patient characteristics

Predictors related to patient characteristics are associated with the risk of late recurrence. In fact, they represent the substrate for the risk of hepatocarcinogenesis and generally do not correlate with the aggressiveness of the primary tumor, which is strongly associated with early recurrence instead. However, some studies have shown a certain impact of the patient's basal phenotype on HCC recurrence that occurs in the first 2 years after treatment (Table 1).

In this regard, the presence of liver cirrhosis and the degree of hepatic dysfunction are the factors that most increase the risk of early recurrence after curative treatment[37,41]. In the models used for risk stratification in patients undergoing hepatectomy, the degree of hepatic dysfunction is quantified by the Child Pugh score[56,57], the model for end-stage liver disease[41] or by the Albumin-Bilirubin score[37,58]. The latter in particular seems to have better discriminatory power in this setting since it helps to further stratify stage A of the Child-Pugh score, which includes almost all patients undergoing LR[58]. Total bilirubin levels, an expression of the degree of hepatic dysfunction, also correlate with the risk of early recurrence[42]. Furthermore, according to Chan *et al*[37] male patients undergoing hepatectomy show a higher risk of early recurrence than female patients.

The predictive value on outcomes (mortality and recurrence) of the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) in HCC patients has been extensively studied. These scores are an expression of the systemic inflammatory response, a known preoperative risk factor for HCC outcomes[49,59,60]. High PLR has been shown to predict lower overall survival (HR: 1.63, 95%CI: 1.34-1.98) and earlier HCC recurrence (HR: 1.52, 95%CI: 1.21-1.91)[59]. Similar results were also highlighted for NLR[49,60]. In addition, both the NLR and PLR were identified as independent risk factors for predicting overall survival and recurrence-free survival (RFS) in HCC patients[59,60].

In recent years, a heated scientific debate has been carried out on the hypothesis of an increased risk of HCC incidence and recurrence in chronic HCV patients treated with DAAs. Unlike interferon (IFN)-based therapeutic regimens, which have been proven to reduce the incidence and recurrence of HCC in cases of sustained virological response[61], some data indicated that after DAA therapy HCC risk may remain high[4,62]. In particular, the retrospective analysis of some patient cohorts revealed an unexpectedly high rate of early HCC recurrence in patients undergoing antiviral treatment with DAAs [62,63]. It has been hypothesized that the rapid fall in viral load induced by DAAs could lead to an altered immune surveillance, promoting the growth of already existing cancer clones[4]. However, the higher number of patients eligible for antiviral treatment with DAAs could influence the apparent raise in early recurrence rates. In fact, due to the manageability and safety profile of these therapeutic regimens, a larger number of patients with advanced liver disease at high risk of HCC (*e.g.*, liver cirrhosis, advanced age, multiple morbidity) have undergone antiviral treatment than those who were candidates for IFN-based schemes. Subsequent prospective studies underlined that although the rates of early HCC recurrence remain high in patients who have obtained viral clearance by DAAs, these rates are comparable to those reported in the literature for patients not treated with these regimens[64-66].

Waziry *et al*[67], in a meta-analysis, concluded that there is no evidence that DAA therapy was associated with higher HCC recurrence and that the available data do not suggest differentiated surveillance pathways for HCC among patients receiving IFN-free therapeutic regimens or receiving DAA treatment. However, the heterogeneity of the studies included in this analysis limited the power of

its results. Although there is no conclusive evidence at the moment in favor or against an increased risk of early HCC recurrence in patients undergoing treatment with DAAs, it is clear that the risk of early HCC recurrence in patients with HCV-related cirrhosis remains high despite viral eradication.

The latest guidelines of the European Association for the Study of the Liver (published in 2018) suggested a more intensive surveillance strategy (3-4 mo imaging intervals for the first 2 years that can be extended to 6-mo intervals thereafter) in this patient setting[4]. However, the updated literature available after the publication of these guidelines almost unanimously confirmed that DAA therapy was not associated with an increase in early HCC recurrence rates and that both recurrence rates and tumor patterns after initiation of antiviral therapy did not differ between patients who received IFN-based or IFN-free therapy[68-76]. On the contrary, there is now increasing evidence of a reduction in the risk of early (as well as late) HCC recurrence after viral eradication achieved by DAA[77-83].

Predictors of early recurrence related to HCC treatment

The choice of treatment for HCC depends on the characteristics of the patient (in particular the residual liver function and the performance status) and the neoplasm characteristics (size, single or multifocal, vascular invasion, distant metastasis) as well as the indication and eligibility for LT.

In terms of recurrence risk, no conclusive data are available in the literature about which type of loco-regional treatment is more advantageous. In the absence of liver cirrhosis, LR probably offers the best prognostic prospects, with an early recurrence rate of approximately 10%[36]. Including patients with liver cirrhosis, the early recurrence rates of post-hepatectomy HCC reported in the literature range from 25% to 50%[34,84,85]. Metachronous HCC, new postoperative lesions and intrahepatic metastasis from primary HCC are in fact relatively common following hepatectomy[42]. In this regard, the resection margins after hepatectomy appear to be closely related to the risk of recurrence. In particular, neoplastic invasion of the resection margins represents a significant predictor of the risk of recurrence[44]. A resection margin < 1 cm also seems to be an independent risk factor for early HCC recurrence[43,86]. Some evidence also suggests that a non-anatomic hepatic resection (resection or enucleation without regard to sectoral structure) could lead to an increase in early recurrence rate[45]. In fact, an anatomic resection (resection of the entire liver parenchymal tissue supplied by the portal venous system draining the HCC tissue, independent of margin length) could hinder cell dissemination, reducing tumor cell flow throughout the portal circulation and consequently reduce the risk of intrahepatic metastasis and improve RFS. However, the absence of randomized clinical trials comparing anatomical and non-anatomical hepatic resection do not provide conclusive indications[87].

As previously mentioned, a maximum tumor diameter greater than 2.6 cm (according to Zhu *et al*[42]) or 3.0 cm (according to Jung *et al*[36]) before a curative LR is predictive of a high rate of early recurrence. The risk of early HCC recurrence with a diameter greater than 2.6 cm appears 4.77-fold higher than neoplasms with a diameter ≤ 2.6 cm[42]. Furthermore, high preoperative total bilirubin levels also correlate with an increased risk of recurrence[42]. In particular, this risk would be increased by 6% for every 1 mmol/L increase in preoperative bilirubin. Chan *et al*[37] in a large multicenter study provided pre- and post-hepatectomy risk stratification models (ERASL-pre and ERASL-post, respectively) with the ability to identify three early recurrence risk classes, with a 2-year malignancy free survival rate of 64.8%, 42.5% and 20.7% in low, intermediate and high risk patients, respectively. In these models male sex, large tumor size, multifocal tumor, high Albumin-Bilirubin score and high serum AFP represented the factors closely related to early recurrence after LR. Ideally, these models, once validated, would optimize the therapeutic strategy for patients with high baseline risk of early recurrence after hepatectomy (revaluating the possibility of LT or providing adjuvant therapy) and intensify the surveillance program.

Compared to LR, RFA treatment shows significantly higher recurrence rates[41,88,89]. In particular RFA results in shorter overall survival and time to recurrence (5-year overall survival of 71.1%, 5-year recurrence time of 63.8%) than LR (5-year overall survival of 61.1%, 5-year recurrence time of 71.7%) [88]. The HR for death (0.84, 95%CI: 0.74-0.95) and recurrence (0.74, 95%CI: 0.68-0.79) was significantly lower in patients undergoing LR than in those undergoing RFA. Recently, Lee *et al*[41], through a retrospective study comparing the two methods, showed that in patients with BCLC stage A HCC treatment with RFA was associated with a 1.8 times higher probability of HCC recurrence than hepatectomy (95%CI: 1541-2216). While confirming higher overall survival rates after LR compared to RFA, other data were unable to demonstrate significant differences in RFS between the two methods [90]. Finally, similar to what was shown for LR, an ablation margin ≥ 10 mm away from the tumor lesion guarantees better outcomes and a lower rate of recurrence compared to an ablation margin ≥ 5 mm and < 10 mm[91].

PREDICTIVE FACTORS OF LATE RECURRENCE

Conventionally, HCC recurrence is defined as late when it occurs 2 years after treatment of the primary tumor. Late recurrence is generally related to de novo development of the neoplasm and does not depend on the characteristics of the previous tumor. About 90% of late relapses consist in exclusively

intrahepatic localization, whereas the remaining cases show both intrahepatic and extrahepatic localizations[92]. Therefore the risk factors are mainly related to underlying liver disease (Table 2).

Predictors of late recurrence related to patient characteristics

Among the predictive factors of late recurrence related to the basal characteristics of the patient, it is easily understood that the main predictive factor is the presence of liver cirrhosis, a fertile substrate for hepatocarcinogenesis[93-96]. In particular, the presence of liver cirrhosis increases the risk of late HCC recurrence by 3 or 4 times[41,93]. In fact, liver cirrhosis is a pre-malignant condition that due to an accelerated hepatocyte turnover induced by the chronic inflammatory state promotes the accumulation of gene aberrations and cell transformations. The high rate of gene errors and the subsequent uncontrolled cell proliferation therefore favor the development of HCC[97]. However, patients with liver cirrhosis undergoing curative treatment for HCC show a late recurrence rate significantly higher compared to de novo incidence rate in patients with no history of prior HCC[41]. This implies that the presence of liver cirrhosis is not the only factor that elicits the cancer risk in patients with late recurrence.

As highlighted by Lee *et al*[41], Xu *et al*[92] and Yang *et al*[93], the age and sex of the patient also contribute to the late recurrence risk stratification. Older and male patients show an increased risk of late HCC recurrence after curative treatment. The sex difference in the late recurrence rate could be explained by the potential protective role of estrogen on the development of HCC[98,99], resulting in more than 3 times higher probability of late recurrence in men compared to women[93].

The predictive role of liver stiffness (LSM) in the risk of HCC occurrence is well documented in the literature[100,101]. Since occurrence and late recurrence show similar pathogenesis (de novo hepatocarcinogenesis), it seems reasonable to believe that LSM can also represent a risk factor for late recurrence. In this regard, Jung *et al*[102] had already highlighted how the presence of liver cirrhosis assessed by LSM ≥ 13.5 kPa was associated with an increased overall risk of recurrence. More recently, Marasco *et al*[43] demonstrated that LSM but even more the splenic stiffness (surrogate for the degree of portal hypertension) were associated with an increased risk of late HCC recurrence. Late RFS was significantly different according to the splenic stiffness cutoff of 70 kPa.

These data find further evidence in the literature. Fang *et al*[40] in fact showed how the splenic volume (evaluated through automated volumetry software from preoperative computed tomography images) correlated independently with the probability of HCC recurrence 2 years after hepatic resection. In particular, for each 1 mL increase in splenic volume, the risk of late recurrence increased by 0.3%. In addition, patients with high splenic volume (> 165 mL) showed a lower 5-year RFS (5-year RFS 36%) than patients with low splenic volume (< 165 mL, 5-year RFS 71%). The correlation between stiffness/splenic volume and the risk of late HCC recurrence probably comes from the close association with the severity of liver disease and portal hypertension, both involved in hepatocarcinogenesis. The correlation that some studies showed between the risk of late recurrence and platelet counts or the presence of esophageal varices, which are also surrogates of the degree of portal hypertension and severity of liver damage[43,103,104], is therefore easily explained.

In patients with chronic HBV infection, the presence of viral replication increases the risk of late recurrence[105-108]. In particular, patients undergoing LR for HCC with high preoperative HBV-DNA levels show lower median overall survival and RFS compared to those with low viral load[106]. Patients with high viremia are characterized by higher Ishak inflammatory and fibrosis scores, favoring hepatocarcinogenesis[105]. The liver inflammatory activity induced by the high viral load may lead to necrosis and regeneration of hepatocytes, increasing the rate of gene errors and neoplastic transformation.

Recently, it was hypothesized that a high viral load (HBV-DNA > 10^4 copies/mL) was a risk factor also of early recurrence[109]. In fact, high preoperative levels of HBV-DNA increased the risk of microvascular invasion by about 40%[110]. In patients with high preoperative viral load, the initiation of antiviral treatment after surgical resection reduced both early and late HCC recurrence rates[106,109,110]. However, a similar effect on late recurrence rates was also found in patients with low preoperative viral loads[111]. Despite comparable efficacy in obtaining virological response, recent evidence suggested that tenofovir disoproxil fumarate (TDF) treatment was associated with a significantly lower risk of both HCC occurrence[112] and early and late recurrence[113] compared to treatment with entecavir (ETV). In particular, the HCC recurrence rate at 5 years from surgical resection was 33.6% in patients treated with TDF and 44.5% in those treated with ETV[113]. Unlike ETV, TDF induced the synthesis of high serum levels of IFN-lambda 3 (IFN-λ3)[114], which has been shown to exert a strong antitumor activity[115,116]. The antitumor effect of IFN-λ3 induced by TDF would therefore be additive to the capacity of breaking down viral replication and turn off necroinflammatory activity, which is common to both nucleoside (ETV) and nucleotide (TDF) analogs.

Increasing evidence is also available on the predictive role of hepatitis B surface antigen (HBsAg) levels on the rate of late HCC recurrence. In patients undergoing LR, preoperative HBsAg levels > 200 IU/mL were independent predictors of late recurrence (HR: 1778)[84,103]. If the role of the predictor is well defined, there is nevertheless a significant heterogeneity of the cutoffs used. Huang *et al*[86] in fact highlighted how in patients with low viral load the risk for HCC recurrence significantly increased with HBsAg levels ≥ 1000 IU/mL (5-year RFS rate of 46.1% in HBsAg ≥ 1000 IU/mL group *vs* 34.1% in HBsAg < 1000 IU/mL group). According to Sohn *et al*[107], HBsAg levels ≥ 4000 IU/mL were associated

Table 2 Predictors of late hepatocellular carcinoma recurrence after curative treatment

Predictors related to HCC characteristics	Ref.	Predictors related to patient characteristics	Ref.	Predictors related to treatment	Ref.
Size > 5 cm	[92]	Liver cirrhosis	[32,41,43,84,93-96]	RFA (*vs* LR)	[41,88,117-119]
Multifocality	[92,93]	Old age	[41,92]		
Microvascular invasion	[92]	Male sex	[93]		
AFP > 400 μg/L	[94]	LSM	[43,102]		
		Portal hypertension			
		SSM (> 70 kPa)	[43]		
		Splenic volume (> 165 mL)	[40]		
		Platelet count	[43,103,104]		
		Esophageal varices	[43,103]		
		HBV infection			
		High viral load	[105,108]		
		Low viral load	[111]		
		High HBsAg levels	[84,86,103,107]		

AFP: Alpha-fetoprotein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LR: Liver resection; LSM: Liver stiffness; RFA: Radio-frequency ablation; SSM: Splenic stiffness.

with late recurrence after curative resection in HBV-related HCC.

Predictors of late recurrence related to HCC treatment

Several studies suggested that RFA treatment increases the risk of early and late recurrence (HR: 1872; 95%CI: 1290-2717) compared to LR[41,88,117-119]. Furthermore, the overall survival at 3 years and 5 years of patients undergoing LR appeared significantly higher than patients undergoing RFA[117,118]. In particular, the 5-year overall survival rates were 80% *vs* 66%, and 5-year RFS rates were 48% *vs* 18% for LR and RFA groups, respectively[118]. Because the choice of treatment is influenced by both tumor- and liver disease-related factors, some authors hypothesize that the correlation between RFA treatment and the risk of late recurrence could reflect both. In fact, patients undergoing RFA are generally older, have a greater number of comorbidities and a worse degree of liver function than patients undergoing LR. As noted above, these factors are closely related to the risk of both late and early recurrence. Therefore, the higher risk of late recurrence in patients who received RFA compared to those treated with resection could depend on more severe underlying liver disease.

Predictors of late recurrence related to HCC characteristics

Some evidence suggested (which was different from what was expected[95,96]) that factors related to tumor burden (size, multifocality) determined an increase in the risk of late and early recurrence[45,93,105]. Recently, Xu *et al*[92] confirmed in a multicenter retrospective analysis of patients who underwent curative LR for HCC that multifocality, dimensions greater than 5 cm and the presence of satellite nodules or vascular invasion (macroscopic or microscopic) represented independent risk factors of late recurrence. The presence of multifocal HCC at baseline increased the risk of a late recurrence after curative treatment by more than 3 times (HR: 3766, 95%CI: 2287–6201)[93]. In this regard, Cheng *et al*[94] also showed that pre-hepatectomy AFP levels > 400 ug/L were related to the risk of late (as well as early) recurrence. Given the dichotomy between early and late recurrence, these factors should correlate with an increased probability of occult intrahepatic dissemination (and therefore early recurrence) rather than an increased risk of hepatocarcinogenesis (late recurrence). These data suggest that the dichotomy between early and late recurrence is probably not so clear-cut, and pathogenetic mechanisms and clinical features may overlap. Therefore, the temporal criterion alone is not able to discriminate the pathogenesis and aggressiveness of a recurrence of HCC with certainty.

LIVER TRANSPLANT AND HCC RECURRENCE RISK

LT represents the most radical approach for HCC in patients with liver cirrhosis and is able to treat

simultaneously the neoplasm and the underlying liver disease while minimizing the risk of both early and late recurrence when candidate selection is adequate[120]. In fact, a therapeutic strategy that provides for early listing for transplantation and a loco-regional bridging therapy in patients with HCC at high risk of recurrence has proven to be extremely valid and associated with excellent long-term outcomes[121]. The presence of occult extrahepatic dissemination and the persistence of the cause of liver damage (*e.g.*, HBV infection or HBV/hepatitis D virus coinfection) account for the residual risk of early and late HCC recurrence, respectively. Overall, the estimated HCC recurrence rate in patients undergoing LT is between 12% and 20%[22,122,123].

The most important predictor of the risk of early post-transplant recurrence is certainly the tumor burden. Size and number of lesions closely correlate with this risk, although not in a linear way. In fact, in multifocal HCC, starting from three or more lesions, the increase in the risk of recurrence appears to be attenuated[124]. Conversely, this risk is proportional to the size of the tumor. In particular, the recurrence rate increases by 36% for each additional centimeter of HCC diameter[125]. The microvascular invasion is another determining factor in the risk of recurrence and HCC-related death [126,127]. Its presence increases the risk of recurrence by approximately 2.4 times and significantly reduces the 5-year rates of RFS (44% *vs* 64% in the absence of microvascular invasion)[126]. Similarly, the histological finding of poorly differentiated (grade 3 or 4) HCC determines an increased risk of recurrence (39.3% *vs* 13.0% for grade 1 and 2 tumors) and reduction of 5-year RFS (39.9% *vs* 57.7%)[127]. In this setting, the prognostic role of AFP is also relevant with an inverse relationship with the post-transplant survival rate[128]. An increase in AFP greater than 7.5 ng/mL per month is associated with the presence of microvascular invasion and is predictive of post-transplant recurrence[129].

Beyond the characteristics and aggressiveness of the tumor, the etiology of liver disease also affects the rate of recurrence, especially late recurrence. In particular, the highest recurrence rate was found in patients with chronic HBV infection (18%) compared to other etiologies (11%, 10% and 8% for HCV, alcoholic liver disease and nonalcoholic steatohepatitis, respectively). In this regard, preliminary evidence suggested that an increase in RFS for HCC patients following LT can be obtained by administering anti-HBV prophylaxis and/or anti-HBV immunoglobulins[130].

Finally, some comorbidities may also influence the risk of recurrence in HCC patients undergoing LT. In particular, obese patients show a significantly higher frequency of microvascular invasion and recurrence rates and lower RFS than normal weight patients[131,132]. This correlation could be explained by a more pronounced tumor neoangiogenesis in obese patients, secondary to the increased expression of vascular endothelial growth factor. Furthermore, in obese patients the reduction of adiponectin levels and the simultaneous increase of leptin induced a pro-oncogenic state and stimulated neoplastic proliferation[131,132].

The central theme in managing the risk of recurrence is the selection of patients eligible for LT. Currently, the strongest evidence identifies the Milan criteria as the best strategy to optimize the selection of LT candidates, stratifying the risk of early recurrence, strongly correlated to tumor size and number of focal lesions[22]. These criteria suggest the presence of single tumors ≤ 5 cm or multiple tumors ≤ 3 nodules sized ≤ 3 cm, without vascular invasion and/or extrahepatic involvement as boundaries for transplant eligibility[24]. The application of these criteria guarantees a post-transplant survival rate comparable to that of patients undergoing transplants for non-neoplastic causes[133]. On the other hand, patients undergoing organ transplantation beyond the Milan criteria show significantly higher HCC recurrence rates than patients within these criteria[22].

However, growing evidence suggests that these criteria, developed in 1996, may be excessively restrictive to date, leading to the exclusion of a subgroup of patients who could benefit from transplantation[120]. Yao *et al*[134] showed that expansion of the tumor size limits (solitary tumor ≤ 6.5 cm or ≤ 3 nodules with the largest lesion ≤ 4.5 cm and total diameter ≤ 8 cm-UCSF criteria) does not adversely impact survival post-transplant. Subsequently, the same authors validated these criteria, confirming a 5-year RFS rate of 81%[135]. The extension of the Milan criteria suggested by Yao *et al*[135] made the 5%-20% of previously excluded patients eligible for LT, guaranteeing comparable long-term survival rates. Also Mazzaferro *et al*[124] attempted to overcome the previous limits through the up-to-seven criteria: HCC with seven as the sum of the size of the largest tumor (in centimeters) and the number of tumors. They showed that in the absence of microvascular invasion patients who met these criteria demonstrated survival rates comparable to patients who met the original Milan criteria (5-year overall survival of 71.2%). Conversely, the presence of microvascular invasion doubled the likelihood of recurrence and significantly reduced the overall post-transplant survival rates in these patients (up-to-seven patients 5-year overall survival of 53.6% *vs* Milan criteria patients 5-year overall survival of 73.3%). Microvascular invasion was observed in 16.6% of patients who met the Milan criteria and in over half of patients beyond the Up-to-seven criteria[124].

Recently, Mazzaferro *et al*[136] attempted to identify factors associated with HCC-related deaths of patients who underwent LT and to provide a predictive model of survival. The number of lesions and their size, as well as the levels of AFP, were significantly associated with HCC-specific deaths. To ensure an HCC-specific post-transplant survival rate of at least 70%, the authors suggested that the sum of the number and size of tumors (in centimeters) should not exceed 7 when the level of AFP was < 200 ng/mL, should not exceed 5 when the level of AFP was 200-400 ng/mL and should not exceed 4 when the level of AFP was 400-1000 ng/mL. Several studies confirmed the predictive value of pretransplant

AFP levels for HCC recurrence[22,137]. A similar role has also been demonstrated for PIVKA-II (5-fold increased risk for recurrence after transplantation)[138].

An approach independent of the size of the HCC and the number of lesions has been hypothesized through the "extended Toronto criteria"[137]. The eligibility for LT included tumors that do not have extrahepatic spread and/or macrovascular invasion and that have a low histological grade on preoperative biopsy and the patient enjoys a high performance status. The post-transplant survival rates in this case were shown to be independent of the patient's status within or beyond the Milan criteria (5-year overall survival of 69% and 78% for patients who met or did not meet the Milan criteria, respectively, $P = 0.3$) and therefore independent of the size of HCC and the number of lesions[139].

Furthermore, growing evidence suggests that an effective and sustained tumor downstaging with locoregional, surgical or systemic therapies from beyond to within the Milan criteria favorably impact overall and tumor-free survival[25,140,141]. In particular, the success rate of downstaging reported in the literature was higher than 40%[142]. Patients eligible for LT who met the Milan criteria after HCC downstaging showed 5-year tumor event-free and overall survival of 76.8% and 77.5%, respectively, compared to 18.3% ($P = 0.003$) and 31.2% ($P = 0.035$) of patients who underwent standard of care (non-transplantation therapies)[25]. Regardless of the Milan criteria, in patients with HCC eligible for LT, a pretransplantation loco-regional therapeutic approach (neoadjuvant, thermal ablation) reduced the risk of recurrence[4].

CONCLUSION

HCC is currently a potentially curable disease. However, recurrence rates still appear to be extraordinarily high. Although several factors are involved, the identification of predictive factors for both early and late HCC recurrence could optimize treatment strategies (LT, LR, thermal ablation) and surveillance. High-risk patients should be referred to treatments with the lowest recurrence rate (*e.g.*, LT [121]) and/or combination therapies, as well as intensive surveillance programs. The combination of multiple treatments or use of adjuvant or neoadjuvant therapeutic schemes (*e.g.*, immunotherapy) could reduce the recurrence rate and improve overall survival[143]. Research should therefore aim to validate these combined therapeutic strategies, particularly in patients with strong pre- and postoperative predictors of early or late recurrence.

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Country/Territory of origin: Italy

ORCID number: Riccardo Nevola 0000-0003-3320-3878; Ernesto Claar 0000-0002-0819-6023; Domenico Cozzolino 0000-0003-2009-5193; Ferdinando Carlo Sasso 0000-0002-9142-7848; Aldo Marrone 0000-0001-7329-4159; Luigi Elio Adinolfi 0000-0001-8453-4912; Luca Rinaldi 0000-0002-6541-3821.

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REVIEW

Functional constipation in children: What physicians should know

Duc Long Tran, Palittiya Sintusek

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Duc Long Tran, Thailand and Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Bangkok, Thailand

Duc Long Tran, Faculty of Medicine, Can Tho University of Medicine and Pharmacy, Can Tho City 9000, Viet Nam

Palittiya Sintusek, Thai Pediatric Gastroenterology, Hepatology and Immunology Research Unit, Division of Gastroenterology, Department of Pediatrics, King Chulalongkorn Memorial Hospital and Thai Red Cross, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Corresponding author: Palittiya Sintusek, MD, PhD, Doctor, Lecturer, Thai Pediatric Gastroenterology, Hepatology and Immunology Research Unit, Division of Gastroenterology, Department of Pediatrics, King Chulalongkorn Memorial Hospital and Thai Red Cross, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Pathumwan Subdistrict, Bangkok 10330, Thailand. palittiya.s@chula.ac.th

Abstract

Functional constipation (FC) is considered the most common functional gastrointestinal disorder in children with a pooled global prevalence of 14.4% (95% confidence interval: 11.2-17.6) when diagnosed based on the Rome IV criteria. Its pathophysiological mechanisms are thought be multifactorial and complicated, resulting in difficult management. Currently, the most effective medication, when used in parallel with toilet training, is osmotic laxatives. Children's adherence to medication and parental concern regarding long-term laxative use are the main contributors to treatment failure. Recently, novel therapies with a high safety profile have been developed, such as probiotics, synbiotics, serotonin 5-hydroxytryptamine 4 receptor agonists, chloride channel activators, and herbal and transitional medicines; nonetheless, well-designed research to support the use of these therapies is needed. This review aims to focus on multiple aspects of FC in children, including global prevalence, pathogenesis, diagnostic criteria, tools, as well as conventional and novel treatment options, such as non-pharmacological management, including adequate fiber and fluid intake, physiotherapy, or neuromodulators. We also report that in very difficult cases, surgical intervention may be required.

Key Words: Constipation; Children; Laxative; Treatment; Toilet training; Herbal

Core Tip: Functional constipation (FC) is a typical symptom of functional gastrointestinal disorders in children and its prevalence is high worldwide. Since the pathophysiology of FC in children is associated with stool withholding behavior, successful toilet training in combination with osmotic laxatives is crucial for the treatment childhood FC. Additionally, promising and innovative drugs can also aid in treatment success.

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INTRODUCTION

Functional constipation (FC) is considered a great disease burden in children that needs early screening and detection. The prognosis of FC is better in children with prompt and proper management. General physicians and pediatricians are usually the first person who take care these children hence understanding the pathophysiology of FC can lead to the proper management and satisfied outcome. In this chapter, the content will be covered all aspect the physician should know about FC for better patient care.

EPIDEMIOLOGY

Globally, up to 25% of visits to pediatric gastroenterologists and 3% of all general pediatric outpatient visits are due to FC[1]. It is difficult to determine the true prevalence of FC in children due to the heterogeneity of the studies in terms of target population sampling, diagnostic criteria, participant ethnicity and environment, method of data acquisition, and life style and psychological factors among others[2]. The primary reasons for the global diversity in prevalence among published studied may be due to the lack of agreement on diagnostic standards and cultural differences[3]. A systematic review and meta-analysis published by Koppen *et al*[4] reported that the worldwide prevalence of FC according to the Rome III criteria was 9.5% [95% confidence interval (CI): 7.5%-12.1%], with significantly more American and European children being affected than Asian ones. Additionally, geographical region, diet, and exposure to traumatic life events were linked to FC in children.

We determined that the pool global prevalence of FC in children was 14.4% (95%CI: 11.2-17.6) using the Rome IV criteria. According to continent, Africa had a highest prevalence of constipation (31.4%), followed by America (12.1%, 95%CI: 9.1%-15.1%), Europe (8.3%, 95%CI: 3.7%-12.9%), and Asia (6.2%, 95%CI: 1.3%-11%). Moreover, the factors significantly associated with FC from these studies are summarized in Figure 1 and Table 1.

NORMAL DEFECATION AND FC

Frequency of toileting habits in infants and children varies with age. To reduce parental worry and prevent needless testing and treatment, knowing the typical toilet routines for all age groups is important[5]. The frequency of stool passage per day gradually decreases from more than four times per day during the first week of life to three times per day at 4-6 wk of life and one to two times per day by the age of 4 years[6,7]. Healthy infants who are exclusively breastfed will have infrequent stool passage at 1-2 mo of life, with a mean duration of 6 days per stool passage (2-28 d per stool passage) without any abnormalities[8]. This condition will normalize at a mean age of 3.9 mo (range 1-7 mo)[9]. Hence, if the stool is soft, the infrequent stool passage in this age group requires neither intervention nor treatment. From the age of five, the majority of children pass stools daily or every other day without straining or withholding[3]. In early newborns, the average intestinal transit time is around 8.5 h, whereas intestinal transit times after puberty range from 30 to 48 h[5].

FC in adults had been first defined in 1999 according to the Rome II criteria and was mostly based on expert opinion. The diagnostic criteria of FC in children was subsequently established and integrated in Rome III criteria by Rome foundation in 2006. In 2016, the Rome III criteria had been replaced by the Rome IV criteria, with only minor changes being made as shown in Table 2[10]. Children who are not yet toilet trained do not need to include fecal incontinence (FI) and clogged toilet in the diagnostic criteria. In addition, the duration of symptoms in children had been changed from 2 mo in the previous Rome III criteria to 1 mo in the current Rome IV criteria[10,11] to promote early recognition and timely

Table 1 Prevalence of functional constipation in children according to Rome IV and factors associated with functional constipation

Ref.	Country	Population	Sample size	Age	Method of data collection	Prevalence of FC, *n*	Factors associated with FC
Huang *et al* [139], 2021	China	4 community hospitals in Jinhua and Shanghai	2604	0-4 yr	Guardian interview	92 (3.5%)	Vaginal delivery (OR = 0.01, 95%CI: 0.00-0.17), forceps delivery (OR: > 999, 95%CI: 154 to > 999)
Chew *et al*[140], 2021	Malaysia	A well child clinic, University Malaya medical center	534	1-12 mo	Guardian interview	6 (1.1%)	NA
Ibrahim *et al* [141], 2020	Egypt	Randomly schools in Cairo	1082	4-18 yr	NA	91 (8.4%)	NA
Khayat *et al* [142], 2021	Saudi Arabia	Public survey (random) from Western	317	3-18 yr	Questionnaire by Google form/links share on social apps	15 (4.7%)	Guardian characteristics, family income, gender, age, development, previous covid ì nfection ($P > 0.05$)
Benzamin *et al* [29], 2022	Bangladesh	Schools of Dhaka division	707	5-16 yr	Child interview and examination (face to face)	134 (19%)	Female ($P = 0.003$), age ($P = 0.001$), history of FC in siblings/parents ($P = 0.001$), fibers intake ($P = 0.002$), fluid intake ($P = 0.001$), electronic screen time ($P = 0.001$)
Siajunboriboon *et al*[143], 2022	Thailand	2 high schools	1700	14-18 yr	Child interview	138 (8.1%)	Guardian characteristics, BMI, history of allergic diseases ($P > 0.05$)
Chia *et al*[144], 2022	Vietnam	A government hospital and a government kindergarten	1511	0-48 mo	Guardian interview and examination (face to face)	46 (3%)	Male (OR = 3.6, 95%CI: 1.5-8.5), bottle feeding (OR = 18.5, 95%CI: 1.5-219.4), low income (OR = 5.8, 95%CI: 1.7-19.3)
Asia			**8455**			**522 (6.2%)**	
Zwiener *et al* [145], 2017	United States	Online survey	1075	4-18 yr	Guardian interview	144 (13.4%)	NA
Saps *et al*[146], 2018	Colombia	12 schools in 6 cities	3567	8-18 yr	Child interview	382 (10.7%)	NA
Robin *et al*[147], 2018	United States	Online survey panels by CINT, United States	1255	0-18 yr	Guardian interview	186 (14.8%)	NA
Játiva-Mariño E *et al*[148], 2019	Ecuador	1 public and 1 private school	951	8-15 yr	Child interview	137 (14.4%)	NA
Saps *et al*[149], 2020	Colombia	6 outpatient clinics	1334	1-48 mo	Guardian interview (face to face)	281 (15.1%)	NA
Velasco-Benitez *et al*[150], 2020	Colombia	4 public schools	1497	10-18 yr	Guardians interview	194 (13%)	NA
Baaleman *et al* [151], 2021	Colombia	A public school	118	11-18 yr	Child interview	16 (13.6%)	NA
Velasco-Benítez *et al*[152], 2021	Colombia	5 to 8 grade students in Cali	465	10-18 yr	Children interview	52 (28.7%)	NA
Dos Santos *et al* [27], 2021	Brazil	Public parks and school areas	799	5-14 yr	Guardian interview	163 (20.4%)	Sex, type of school ($P > 0.05$)
de Morais *et al* [153], 2022	Brazil	Pediatric private clinics in 5 regions	4560	0-12 mo	Guardian interview	341 (7.6%)	Age 162-248 d (OR = 1.41, 95%CI: 1.01-1.95), prematurity (OR = 1.44, 95%CI: 1.02-2.02)
America			**15621**			**1896 (12.1%)**	
Russo *et al*[21], 2019	Italy	General clinics	214	1 mo-17 yr	Guardian/child interview(face to face)	39 (18.2%)	NA
Vladimir *et al* [154], 2019	Russia	University clinic	300	0-48 mo	Guardian interview	45 (15%)	NA
Steutel *et al* [155], 2020	Belgium, Italy, Netherland	General pediatrics hospital (Belgium, Italy) and well-baby clinic (the Netherlands)	2751	0-48 mo	Guardian interview and examination (face to face)	151 (5.4%)	NA
Campeotto *et al*	France	Private out-patient	1570	0-12	Guardian interview	141 (9%)	NA

[156], 2020		pediatricians and general practitioner		mo	and examination (face to face)		
Alonso-Bermejo *et al*[157], 2022	Spain	A pediatric gastroenterology clinic	574	0-16 yr	Guardian/child interview and examination (face to face)	41 (7.1%)	NA
Beser *et al*[158], 2021	Turkey	9 tertiary Hospital	2383	1-12 mo	Child interview and examination (face to face)	112 (4.7%)	NA
Strisciuglio *et al* [159], 2022	6 Mediterranean countries[1]	Nursery schools, primary schools and secondary schools, randomly	4353	4-18 yr	Guardian interview	475 (10.9%)	NA
Europe			**12145**			**1004 (8.3%)**	
Bellaiche *et al* [160], 2020	10 countries in Africa[2]	Children with gastrointestinal symptoms	10458	0-12 mo	Guardians interview and examination (face to face)	3283 (31.4%)	NA
Africa			**10458**			**3283 (31.4%)**	
All over the world			**46679**			**6704 (14.4%)**	

[1]6 Mediterranean countries: Croatia, Greece, Israel, Italy, Macedonia, Serbia.
[2]10 countries: Algeria, Cameroon, Congo, Gabon, Madagascar, Morocco, Mauritius, Ivory Coast, Senegal, Tunisia.
CINT: A global software in digital insight and research technology; FC: Functional constipation; CI: Confidence interval; OR: Odds ratio; apps: Applications; NA: Not available; BMI: Body mass index.

Table 2 Rome IV criteria for pediatric functional constipation[6,7,9]

Child's age	Diagnostic criteria
< 4 years old	Two or more criteria for at least 1 mo[1]: (1) Two or fewer defecations per week; (2) History of excessive stool retention; (3) History of painful or hard bowel movements; (4) History of large diameter stools; (5) Presence of a large fecal mass in the rectum; (6) At least one episode of fecal incontinence per week after the acquisition of toileting skills; and (7) History of large-diameter stools that may obstruct the toilet in toilet trained children
≥ 4 years old	Two or more symptoms for at least 1 mo in children at least 4 yr[2]: (1) Two or fewer defecations per week; (2) At least one episode of fecal incontinence per week; (3) History of retentive posturing or excessive stool retention; (4) History of painful or hard bowel movements; (5) Presence of a large fecal mass in the rectum; (6) History of large-diameter stool that may obstruct the toilet; and (7) Additional criteria: Without fulfilling irritable bowel syndrome criteria

[1]After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.
[2]After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.

treatment. Although there are some changes of the diagnostic criteria in Rome IV, the prevalence of FC in children was similar using either the Rome IV or Rome III criteria.

FI in children is defined as the involuntary passage of stool into the underwear either as unintentional seepage of small amounts of liquid stools (generally referred to as "soiling" or "leakage") in a child older than 4 years of age or in a toilet-trained child[12,13]. Irrespective of the amount of stool, this is one of the most unpleasant and embarrassing things for a growing child apart from being an upsetting and mentally distressing issue that has a negative impact on children's quality of life. FI was divided into 2 types; retentive and nonretentive FI. It is critical to distinguish between retentive and nonretentive FI given their different etiologies and approaches to treatment[14]. Hospital and community studies have shown retentive FI occurs in constipated children with fecal impaction[1,15,16], whereas the nonretentive type could be found in children with psychological problems[3].

PHYSIOLOGY OF DEFECATION AND PATHOGENESIS

The act of defecation is a process related to the pelvic floor muscles, anal sphincter complex, enteric nervous system, and central nervous system (Figure 2). Normally, children over 18 mo old can initially control defecation through this complicated process, with nearly all of them succeeding in controlling defecation by the age of 4.

The etiology of constipation can be classified into functional and organic causes, which account for 90% and 10% of the cases, respectively[12]. Regarding FC, the pathophysiological mechanism might be multifactorial, including stool withholding behavior, anorectal dysfunctions, diet, physical activity,

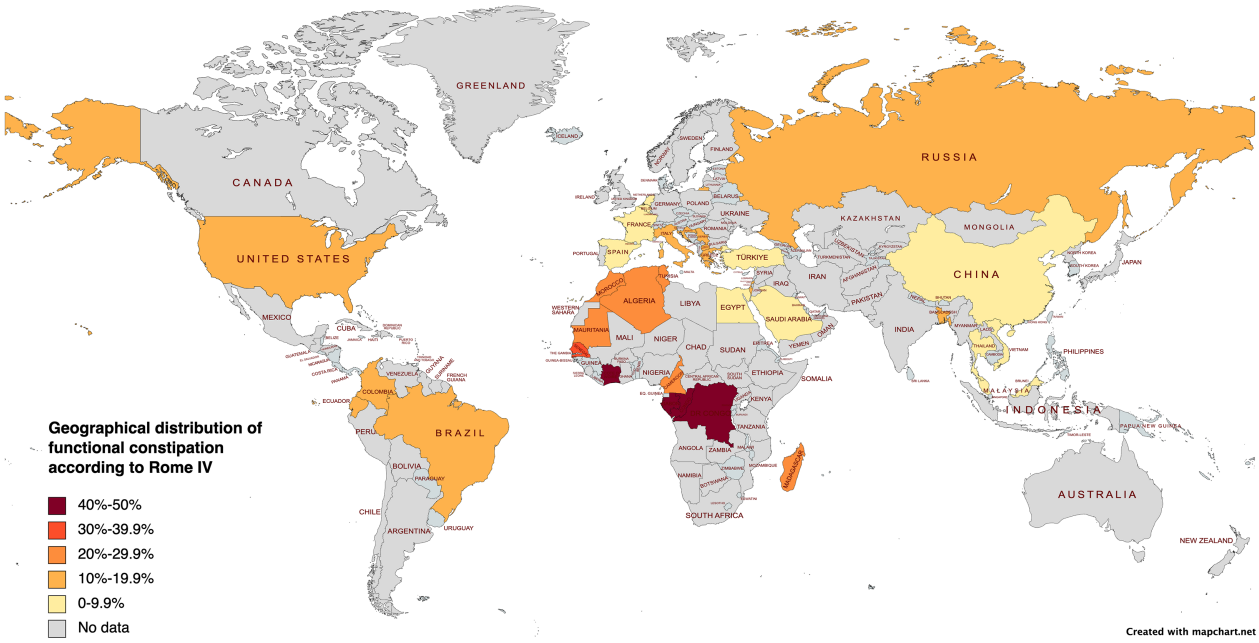

Figure 1 Geographical distribution of functional constipation in children worldwide, according to Rome IV.

genetic predisposition, and psychological issues. Stool withholding behavior is the main pathophysiological mechanism especially in toddlers and young children. Faulty toilet training, painful defecation from hard stool and frequent rectal enema contribute to fear and bad experiences related to defecation, which can cause purposeful or subconscious stool withholding behavior. Instead of relaxing the pelvic floor muscle when feeling the urge to defecate, children will defecate in the standing position and contract the pelvic floor and gluteal muscles, a phenomenon called "retentive posture or defecation in standing position". This behavior promotes the retention of stool in rectum and causes the stool to become lumpier and harder, making it quite difficult to evacuate, due to water absorption by rectal mucosa. This phenomenon leads to a vicious cycle of difficult defecation. Once large stools are retained in the rectum, the rectal wall stretches and develops into a megarectum[17] with decreased sensation to defecate[12]. Moreover, liquid stool can penetrate the hard stool and leak out of the anus, causing fecal soiling (Figure 3). According to pathophysiology, withholding behavior, palpable fecal mass on abdominal examination, and fecal soiling were reported in 37%-91%, 33%-68%, and 33%-77% of in children with FC, respectively[18-21]. Hence, two of the three characteristics were integrated into the Rome IV criteria.

CLINICAL MANIFESTATIONS OF AND EXAMINATIONS FOR FUNCTIONAL AND ORGANIC CONSTIPATION

FC is diagnosed based on symptoms detailed in the Rome IV criteria. However, some examinations may help pediatricians in cases with uncertain symptoms and signs[22]. Moreover, in cases that are difficult to treat or have alarm features (Table 3), examinations to exclude organic cause are necessary. Here we will review the clinical manifestations and examinations that can helpful general pediatricians and specialists diagnose functional and organic constipation (Table 4).

Patient history

When obtaining the medical history of children, it is important to inquire about when the child had their first bowel movement after delivery. Normally, during the first 24 h of life, more than 90% of term newborns pass meconium[23,24]. This period may be longer in preterm infants due to the delayed maturation of the intestinal motor function[25]. If the passage of meconium is delayed after birth, worrisome diseases, such as Hirschsprung's disease (HD) and cystic fibrosis, should be excluded[6,26]. Age of onset, frequency, consistency and size of the stool, painful or difficult defecation, and presence of blood coating the stool are all crucial details to note when recording a patient's history. In addition, frequent clogging of the toilet might reflect a large fecal mass in rectum. Anal fissures should be examined in children with a history of difficult defecation (Figure 4A) and those with blood coating the stool (Figure 4B) or the toilet paper. It is necessary to gather information regarding incontinence or soiling during the day and night. FI can be mistaken for diarrhea by their guardians[1,6] (Figure 4C).

Table 3 Alarm signs and symptoms of constipation[37]

Alarm signs	Symptoms of constipation
History	Constipation starting in neonatal period, delay pass meconium (> 48 h of life), family history of Hirschsprung's disease
Stool characteristics	Ribbon stools, blood in the stools in the absence of anal fissures
Gastrointestinal features	Bilious vomiting, severe abdominal distension
Back	Sacral dimple, tuft of hair on spine, gluteal cleft deviation
Anus	Perianal fistula, abnormal position of anus, anal scar, absent anal/cremasteric reflex
Neurological features	Decreased lower extremity strength/tone/reflex
Others	Abnormal thyroid gland, fever, faltering of growth

Table 4 Organic causes of constipation[1]

Organic causes	
Abnormalities of colon and rectum	Anal or colonic stenosis. Imperforate anus. Anteriorly displaced or ectopic anus. Cloacal malformations. Chronic intestinal pseudo-obstruction
Systemic disorders	Hypothyroidism. Hypercalcemia. Hypocalcemia. Diabetes mellitus. Panhypopituitarism. Cerebral palsy. Myotonia congenita. Scleroderma. Amyloidosis. Mixed connective tissue disease. Myotonic dystrophy. Progressive systemic sclerosis
Others	Cystic fibrosis. Celiac disease. Heavy metal ingestion (lead, mercury)
Spinal cord abnormalities	Meningomyelocele. Spinal cord tumor. Sacral agenesis. Tethered cord
Neuropathic intestinal disorders	Hirschsprung's disease. Intestinal neuronal dysplasia. Chagas disease. Abnormal muscle of abdomen. Prune belly syndrome. Gastroschisis
Drugs	Opiates. Anticholinergics. Antacids. Antihypertensives. Antimotility agents. Cholestyramine. Psychotropics. Diuretics

Evidence of fecal impaction in children suspected of FI is crucial, and physicians could obtain this information through abdominal palpation of a fecal mass, digital rectal examination, or rarely through plain abdominal radiography in noncooperative children. Importantly, physicians must define how a child defecates. Withholding behaviors are considered the main pathogenesis of FC and have been defined by guardians as defecation in the "standing position". This can also be described as stiffening up, buttock clenching, walking on tip toes, crossing one leg over the other, bracing against furniture, being in the all-fours position or curling up in a ball, and sitting with legs straight out (Figure 4D)[27,28]. Withholding behaviors also help clinicians determine that the constipation should be of functional etiology without any organic problems. Other factors, such as significant life events like a family member's death, the birth of a sibling, difficulties in school, sexual abuse, and others, can contribute to retentive behaviors and FC and should be evaluated in detail[1,6].

Although the abdominal pain caused by FC is typically nonspecific and poorly localized, constipation was the cause of acute abdominal pain in 50% of children who presented for a primary care visit and should be considered in this context[1]. Physicians should identify alarm features (Table 4) and other signs and symptoms, including appetite loss, fever, nausea, vomiting, reduced weight gain, issues with neuromuscular development, and behavioral or psychological problems[6,26,29]. Poddar *et al*[18] reported that children with symptoms such as delayed passage of meconium, growth failure, lack of retentive posturing, and absence of fecal impaction may likely have organic FC. Furthermore, urinary tract infections have been reported in a significant number of children suffering from constipation and FI[30,31]. Dietary and constipation treatment history should be investigated to predict the long-term outcomes of FC in affected children[1,6,32].

Physical examination

Assessing children's physical development through weight and height measurements should be the first step[6]. Abdominal examination should obtain information on the rectal fecal mass, particularly its height above the pelvic brim, through bimanual palpation on either side of the rectus sheath[7]. With careful abdominal and digital rectal examination, fecal masses can be detected in 30%-75% of children with FC[1,18]. The perineum should be examined given that it can reveal important details regarding the anal position, evidence of FI, skin irritation, eczema, fissures, and signs of possible sexual abuse[6].

Measuring the anogenital index (Figure 5) is important given that it might be considered a factor associated with FC. The anogenital index can be calculated using the formula presented below[33-36]. The normal anogenital index in males and females is 0.54 ± 0.03 and 0.40 ± 0.04, respectively[35].

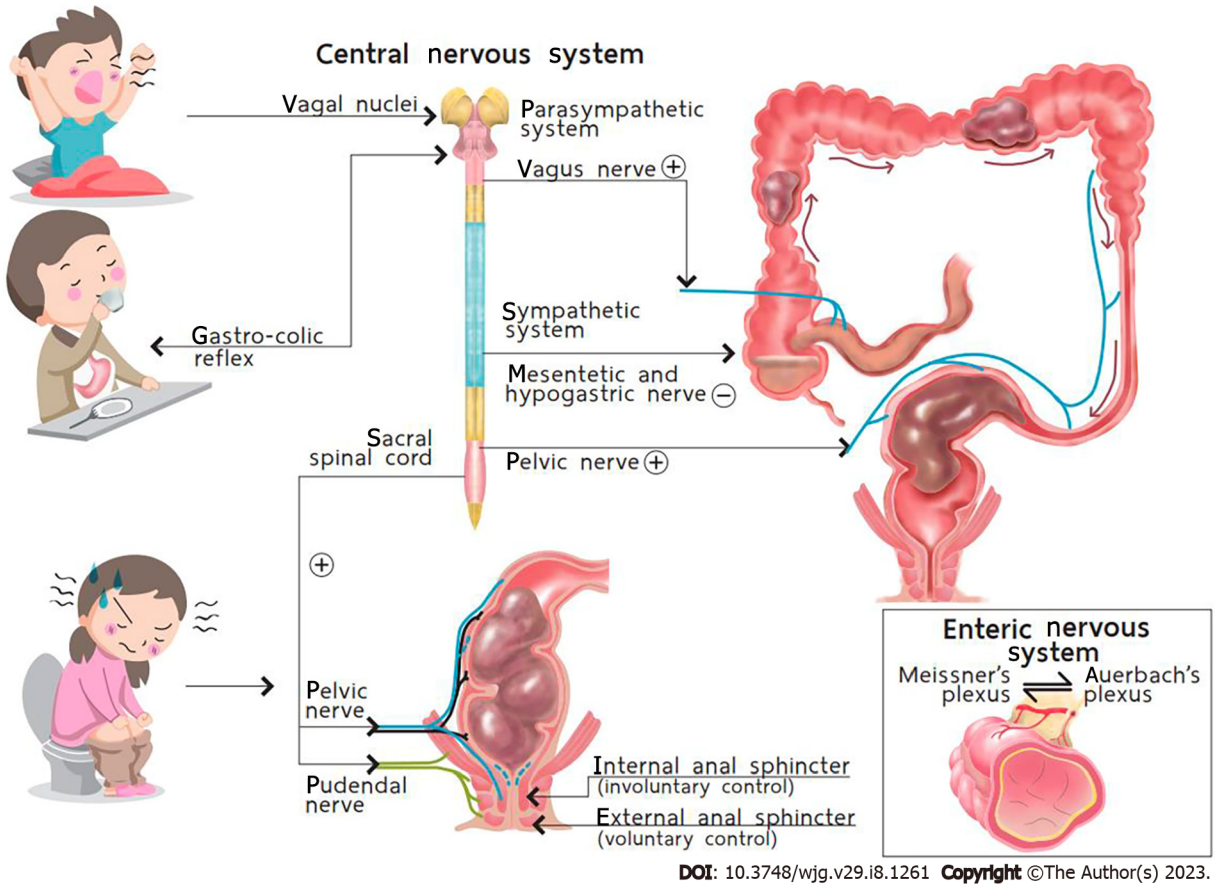

Figure 2 Normal defecation dynamics. Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 417. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).

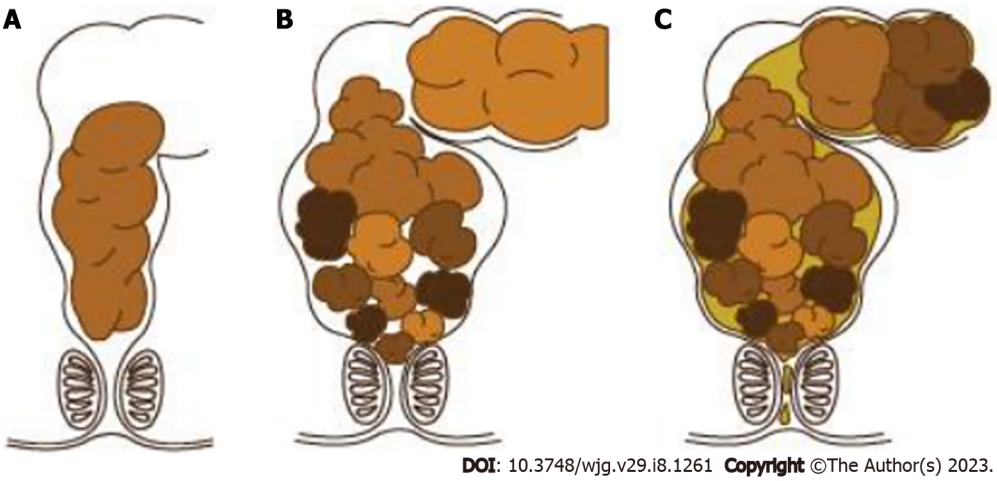

Figure 3 Accumulation of fecal mass in the rectum causes rectal wall distension and fecal soiling. A: Normal stool; B: Massive stool are retained in the rectum from withholding behavior; C: Liquid stool can leak out the anus or fecal soiling. A-C: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

Anogenital index = [vagino/scroto to anal distance (cm) ÷ vagino/scroto to coccygeal distance (cm)] [35].

When the child's history suggests the presence of FC, a digital rectal examination may not be necessary[7,37]. Digital rectal examination should be conducted when children present with red flags, a

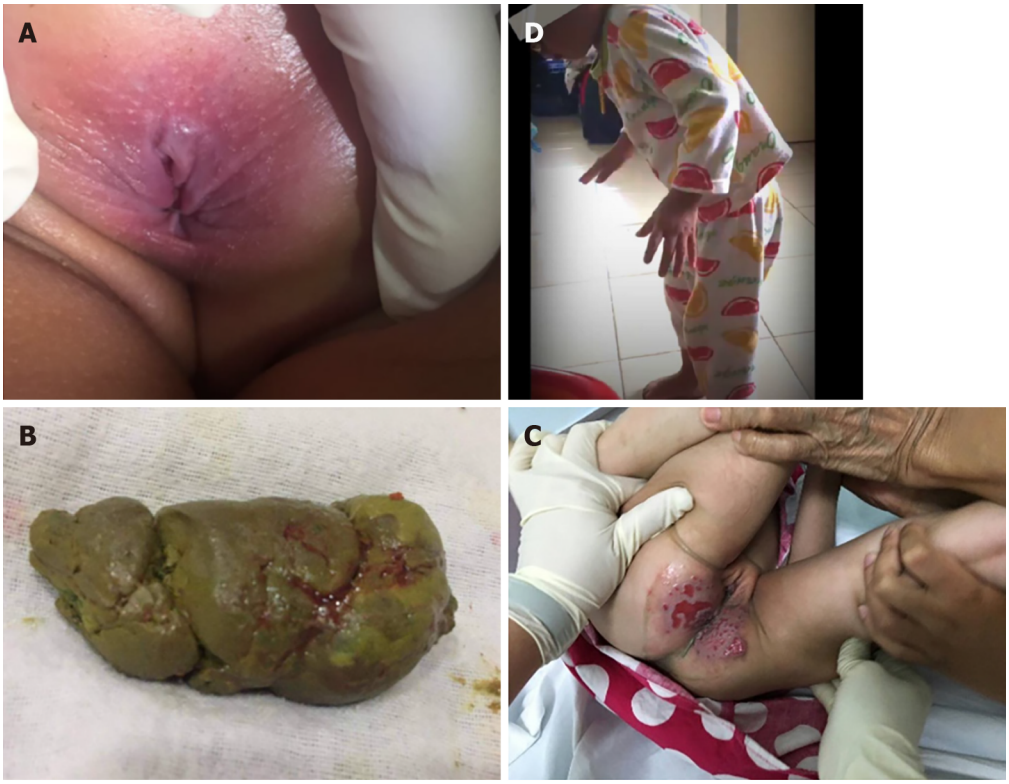

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Figure 4 Clinical manifestations in constipated children. A: Anal fissure usually at the 6 o'clock or posterior parts; B: Blood coated, hard, and lumpy stool; C: Rectal abrasion from fecal incontinence that was misdiagnosed as chronic diarrhea; D: Retentive posture. A-D: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

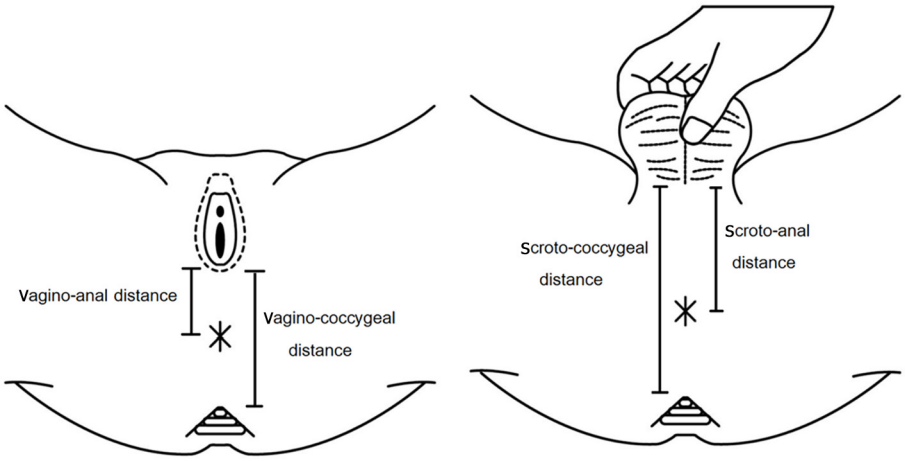

Figure 5 Landmarks for the measurement of the anogenital index. Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

history of delayed meconium passage after birth, intractable constipation, an uncertain diagnosis according to the Rome IV criteria, suspicion of an anatomic problem, and assessment of fecal impaction after disimpaction. Although neurological disease causing organic constipation is very rare, dedicated neurological examination still has merit (Figure 6).

Laboratory examinations

It is necessary to emphasize that FC is a clinical diagnosis based on a detailed medical history and physical examination. The goal of laboratory testing is to determine the presence of a rare organic

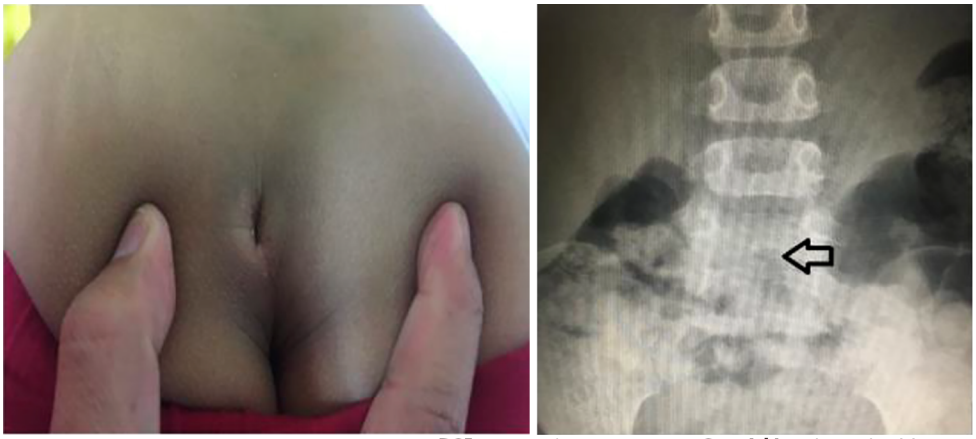

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Figure 6 Sacral dimple and spinal dysraphism on spinal radiography (arrow) in a 5-year-old boy with intractable constipation. A: Sacral dimple; B: Spinal dysraphism.

etiology in children with constipation showing alarm features[37] (Table 3) or confirm the diagnosis of FC in complicated or unclear cases. Hence, further diagnostic interventions are sometimes warranted. Investigations that might be useful for determining organic causes of constipation are described below.

Laboratory testing: Thyroid function and serum calcium tests can be used in children with intractable constipation or chronic constipation who are quite difficult to treat. In countries with a high prevalence of celiac disease and cystic fibrosis, specific tests for these diseases might be considered. Although the prevalence of food allergies in children presenting to tertiary clinics with chronic constipation who are unresponsive to traditional treatment vary from 28% to 78%[38], conflicting data support the use of allergy testing to identify cow's milk protein allergy (CMPA) in constipated children[39-41]. Therefore, according to the recommendations of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NAPSGHAN), routine allergy testing is not recommended in constipated children suspected for CMPA[37]. Oral food avoidance and rechallenge is the gold standard for diagnosing CMPA that manifests with intractable constipation.

Abdominal radiography: Abdominal radiography can be help pediatricians determine the presence fecal masses in some cases where physical examination is limited (Figure 7), such as obese children, patient refusal, or noncooperation, or exclude some causes of acute abdominal pain.

According to systematic reviews, abdominal radiography can identify constipation with a sensitivity and specificity of 60%-80% and 43%-99%, respectively[42,43]. There was inconsistent evidence to support the diagnostic relationship between constipation symptoms and fecal loading in abdominal radiographs from children; hence, the results should be interpreted with caution. In line with this, three scoring systems have been developed, namely the Barr score[44], Leech score[45], and Blethyn score [46]. However, further validation is needed before they can be widely used in clinical practice.

Abdominal ultrasonography: Abdominal ultrasonography can assess stool retention and estimate the size of the rectum and colon based on the supposition that fecal retention is one of the primary characteristics of constipation in both children and adult[6,47]. Given that ultrasound scanning is noninvasive and radiation-free, it is usually used for assessment in primary and secondary clinical care[1,6]. Rectal diameter measurements were correlated with the results of digital rectal examination and therefore seems to accurately assess fecal impaction[43]. Evidence suggests that digital rectal examination might be replaced by ultrasound scanning given that the latter is less unpleasant[48]. Even though there was a good correlation of transverse rectal diameter with FI and long-term constipation[49], the transverse diameter cannot be used to predict fecal impaction or constipation[37]. Furthermore, the results are largely operator dependent, and patient cooperation is also needed.

Radiopaque marker for colonic transit study: Based on the distribution of markers throughout the colon, the radiopaque marker (ROM) for colonic transit time (CTT) study is one method for distinguishing between different types of colonic function, including normal colonic transit, slow-transit constipation, and obstruction of the rectal outlet. Given its accessibility and strong concordance with scintigraphic methods, the ROM for CTT study has become the most popular method for determining both total and segmental CTT[50]. The sensitivity and specificity of the ROM for CTT study were 71% (95%CI: 57%-83%) and 95% (95%CI: 82%-99%), respectively[51]. The mean transit time in healthy persons has been reported to range from 15.6 to 37.7 h, with a review by Southwell *et al*[52] revealing that the normal CTT was < 32 h (upper 95th centile: 54 h).

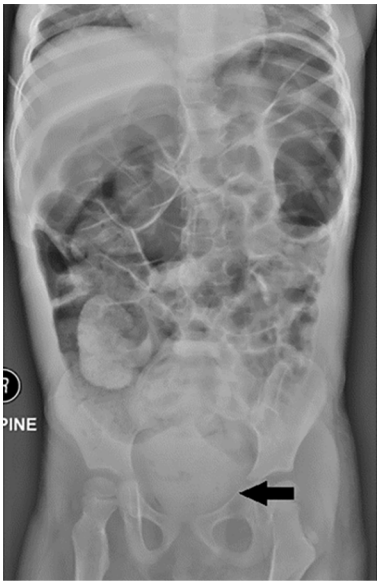

Figure 7 Abdominal radiography of a 3-year-old girl with a chief complaint of chronic diarrhea for 4 mo shows fecal impaction in the rectum. Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

The ESPGHAN and NAPSGHAN recommend that these tests only used to distinguish FC from functional non-retentive fecal incontinence or, where the diagnosis is unclear, provide clarity and allow the selection of alternative diagnostic procedures due to the widespread use of the Rome criteria for the diagnosis of FC and the potential risks caused from repeated radiation exposure from abdominal radiography[37,50].

Colonic manometry: To determine the neuromuscular function of the colon in children with intractable constipation, colonic manometry is regarded as the gold standard[50]. Evidence suggests that colonic manometry is most useful in providing subsequent guidance for further therapy, including pharmacological and surgical management, in intractable constipation. According to a review by the ESPGHAN motility group, high amplitude propagating contractions are the most easily recognizable and reliable motor pattern (Figure 8). They are initiated usually in the proximal colon and expected to stop at the recto-sigmoid junction. However, colonic manometry can be difficult for children given its invasive nature and necessity for general anesthesia. Moreover, age can be a potential limiting factor depending on the size of the catheter and endoscope. Additionally, only a few specialist centers globally offer this test.

Wireless motility capsule: The wireless motility capsule (WMC) is a novel, nonradioactive, and minimally invasive tool for the assessment of colonic motor function. Several investigations have reported on the safety and tolerability of WMC. In recent years, an increasing number of studies have used WMC to diagnose children with functional gastrointestinal diseases (FGIDs)[53,54]. This test could provide information on gastrointestinal motility that is similar to information obtained *via* nuclear medicine gastric emptying time and/or ROM. Moreover, WMC can provide additional information on regional and entire-gut transit[53], which can not only add to our knowledge of colon physiology but also be used as a parameter to tailor treatment[54]. Considering its safety and low invasiveness, the ESPGHAN motility working group recommended that more research be done to assess the effectiveness of the WMC in predicting outcomes among children with intractable constipation[50].

Magnetic resonance imaging: Cine magnetic resonance imaging (cMRI) is a noninvasive tool that uses a high-resolution spatiotemporal approach to facilitate dynamic MRI, which would allow the observation of the gut lumen diameter. The assessment of stomach accommodation and emptying, terminal ileum motility, and the small bowel using cMRI has been documented in the literature[43].

In both adults and children, cMRI can be used to assess colonic motility for various gastrointestinal disorders[55]. However, data on the application of this method in children with intractable FC are currently scarce.

Vriesman *et al*[56] published the first pediatric study comparing the identification of colonic motility patterns on cMRI with that on colonic manometry, proving potential evidence regarding the feasibility of the technique. cMRI has the advantage of being noninvasive, precluding the need for general anesthesia; however, given that this is still a research-based modality, additional studies are required to establish objective and systematic measurements.

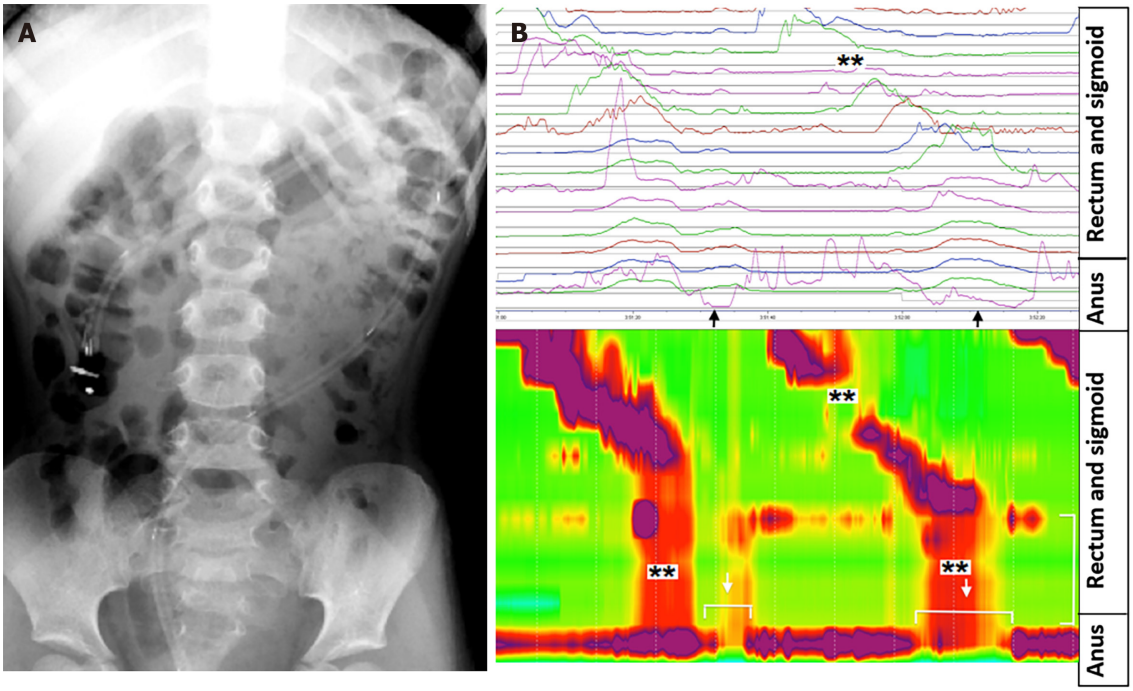

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Figure 8 High amplitude propagating contractions. A: Proper position of the colonic catheter; B: graphical demonstration of abnormal high amplitude propagating contractions (HAPCs) with absent HAPCs at the sigmoid region (**) but preserved colo-anal reflex (arrow). A and B: Citation: Palittiya Sintusek. Chronic constipation. In: Supapitiporn K, editor. Pediatric practice: simple&applicable. Bangkok: Chulalongkorn University, 2021: 96. Copyright ©The Authors 2017. Published by Department of Pediatrics, Chulalongkorn University. The authors have obtained the permission for figure using from Department of Pediatrics, Chulalongkorn University (Supplementary material).

Barium enema and others: A barium enema is used to coat the lining of the colon and rectum in order to create clearer images of the colon (Figure 9A). A contrast enema is often used in the diagnostic workup of HD, in which a transition zone between the aganglionic and ganglionic bowel may be observed in histopathology study[6] (Figure 9B). A 24-h delayed barium enema film could offer comprehensive data on colon transit function in young children, especially those under 4 years old who often cannot undergo CTT study[57]. Moreover, delayed retention of contrast at 48 h provides the strongest negative predictive value to exclude HD. Nonetheless, a limitation of barium enema is that it cannot be used to diagnose ultrashort HD; instead, anorectal manometry is required wherein absence of the anorectal inhibitory reflex is pathognomonic for HD (Figure 9C).

TREATMENT OPTIONS

The goals of treatment include establishing regular defecation (ideally once a day, passing soft stools and without difficulties) and preventing relapses[1,6,58]. Oral laxatives and structured toilet training are the main tools of a successful treatment. FC in children is typically managed across four important phases: (1) Education; (2) Fecal disimpaction; (3) Preventing fecal reaccumulation; and (4) Follow-up.

Education

Education is an important initial step of treatment[59] given its association with adherence and the successful of management of constipated children. In fact, studies by Steiner *et al*[60] and Koppen *et al*[61] reported that 38% and 37% of patients adhered to therapy, respectively. Inconvenience, dissatisfaction with treatment, and emotional impact of symptoms were linked to low adherence, all of which require attention[61].

Therefore, education should include an explanation of the physiological dynamics of defecation; associated factors of constipation; and the related shame, embarrassment, and social issues to the guardians and their children. In particular, the doctor needs to make it clear to the family that withholding behavior is crucial to the pathophysiology of FC. Furthermore, physicians should create a thorough plan to eliminate the frustration of guardians and children and increase cooperation required for prolonged treatment. Moreover, the timing of a successful treatment is frequently unpredictable, and guardians must understand that there is no quick fix for this problem. Recovery is only feasible with a sufficient, frequent, and prolonged care[1,6,59]. In some complex cases, such as in children with

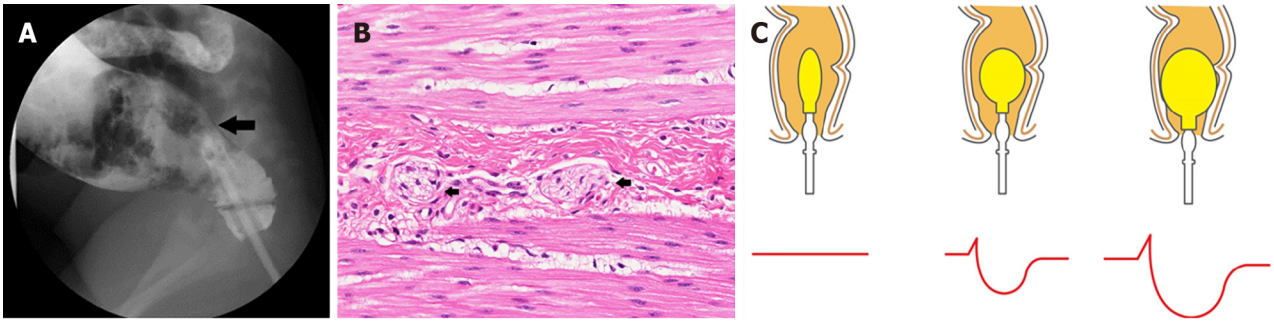

Figure 9 Investigations for organic constipation. A: Transition zone after barium enema in a child diagnosed with Hirschsprung's disease; B: Histopathology revealing no ganglion cells in the nerve plexuses (myenteric plexus) of the muscular propria layer. Arrow shows thick nerve trunk but without ganglion cells; C: Anorectal manometry showing rectoanal inhibitory reflex. A-C: Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 417. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).

intractable constipation or in those suffering from other comorbidities such as urinary problems, it is necessary to involve a multidisciplinary team that includes pediatric specialist nurses, pediatric research nurses, psychiatrists, urotherapists, and urologists for long-term follow-ups. Hence, such children require customized care.

Fecal disimpaction

Fecal disimpaction is crucial before the start of maintenance therapy in order to maximize the success of treatment. If fecal impaction is not eliminated beforehand, maintenance therapy can lead to worsening FI[1,6,59]. Oral drugs, rectal enema, or a combination of both can effectively treat fecal impaction. Two randomized controlled trials (RCTs) showed that polyethylene glycol (PEG) and enemas are equally effective for fecal disimpaction[62,63]. The use of 1-1.5 g/kg/d of PEG with or without electrolytes orally for 3-6 d is recommended as the first-line treatment for constipated children with fecal impaction [37]. However, a RCT study reported that both lactulose and PEG treatment successfully promoted disimpaction and were safe and well tolerated, although PEG achieved disimpaction significantly faster at day 2 (P = 0.001)[64]. Lactulose may be a useful PEG substitution for treating fecal impaction, particularly in areas wherein the availability of PEG is limited.

The oral route is typically less intrusive, better tolerated, and provides children with a better sense of control than rectal enema; however, successful disimpaction can take a few days, with compliance also being an issue. In contrast, the rectal approach (enema) is faster (effect occurs within minutes) but more invasive and traumatic. It may be useful for removing fecal impaction in patients with severe abdominal pain or large fecal mass on abdominal examination. Common side effects of enema include anorectal discomfort and abdominal pain; hence, it should be avoided in scared and resistant children to avoid anal fissures[58]. Recently, a study on the efficacy of olive oil enemas for childhood constipation showed that 79.6% and 66.7% of FC cases in the olive oil and lubricant groups were effectively treated for fecal impaction, respectively[65] (Table 5).

Maintenance therapy

Following disimpaction, maintenance therapy should be started immediately. The goals of maintenance therapy are to produce soft and painless stools, avoid stool reimpaction, and stop the reemergence of stool withholding behavior. This can be accomplished by combining pharmacological and nonpharmacological interventions.

Pharmacological management

Osmotic laxatives: Osmotic laxatives, including PEG, lactulose, and milk of magnesium hydroxide (MOM), are a type of osmotically active ions or molecules that are rarely absorbed in the small intestine. As such, they stimulate water retention in the colon, consequently softening stools.

PEG has been demonstrated to be more effective at increasing bowel movement frequency than lactulose, making it the first option for maintenance therapy in constipated children[66-68] or MOM [69]. However, limited evidence of its the utilization and safety has been available in infants, especially for long-term usage[66,70]. Moreover, data from a 10-year survey revealed that 645 children using PEG 3350 between the ages of 0-21 reported 1564 adverse symptoms. Among these adverse symptoms, 58.75% were neurological or neuropsychiatric, such as anxiety, anger, abnormal behaviors, and others [71]. However, the data source from this survey had significant limitations, including sampling bias, lack of verification, inability to seek clarifications, and lack of follow-up data. Comparing the efficacy of lactulose and MOM, one study found a significant difference in the frequency of stool passage per week,

Table 5 Laxatives used for functional constipation in children[1,6,37,58]

Agent	Child's age	Dosage	Side effects
Osmotic laxatives			
PEG	Any age	0.4-0.8g/kg per day for maintenance; 1-1.5g/kg per day for fecal disimpaction	Diarrhea, bloating, flatulence, nausea, vomiting, abdominal cramps
Lactulose (70% solution)	Any age	1 mL/kg once or twice daily (max 120 mL per day)	Bloating, flatulence, abdominal cramps, fecal, incontinence
Sorbitol (70% solution)	1-11 yr	1 mL/kg once or twice daily (max 30 mL per day)	Bloating, abdominal cramps
	> 12 yr	15-30 mL once or twice daily	
Milk of magnesium	> 2 yr	1-3 mL/kg per day once or twice daily	Abdominal pain, fecal incontinence, hypermagnesaemia, hypocalcaemia, hypophosphataemia (with excess use in children with renal disease)
Stimulant laxatives			
Senna (antraquinone)	> 2 yr	7.5-15 mg/kg per day once daily	Abdominal cramps, idiosyncratic hepatitis, melanosis coli in prolong used, nephropathy, neuropathy, hypertrophic osteoarthropathy
Bisacodyl	> 2 yr	5-10 mg per day once daily	Diarrhoea, abdominal cramps
Sodium picosulphate	4-5 yr	3 mg per day	Nausea, vomiting, bloating, abdominal cramps, diarrhea, headache, taste impairment
	> 6 yr	4-6 mg per day	
Glycerine suppository	< 1 yr	Half for pediatric suppository once daily	Rectal irritation, bloating, abdominal cramps, diarrhea
Rectal laxatives/enemas			
Sodium phosphate	> 1 yr	2.5 mg/kg	Rectal discomfort, diarrhea, abdominal cramps, electrolyte imbalance
Bisacodyl	2-12 yr	5 mg/dose once daily	Rectal discomfort, diarrhea, abdominal cramps, hypokalemia
	> 12 yr	5-10 mg/dose once daily	
Saline enema	Neonate	< 1 kg: 5 mL, > 1 kg: 10 mL	Rectal discomfort, bloating
	> 1 yr	6 mL/kg once or twice daily	
Lubricant			
Mineral oil	> 1 yr	1-2 mL/kg daily (max 90 mL per day)	Rectal discomfort, lipoid pneumonitis

PEG: Polyethylene glycol.

favoring MOM over lactulose (MD: 1.51, 95%CI: -2.63 to -0.39, 50 patients). Besides PEG, lactulose and MOM have been used as second-line drugs, with MOM being very cheap and widely available in some Asian countries such as Thailand[72]. However, the main limitation of MOM is its terrible palatability as opposed to lactulose. The concerning adverse effect of MOM is just only awareness of hypermagnesemia, especially with long-term usage in children with chronic renal disease. Conversely, lactulose can used safely even among preterm infants[73], with the only common adverse effect being abdominal distension. Though PEG is the most effective osmotic laxative for the treatment of function constipation in children, in areas or situations where availability is limited, MOM or lactulose might be used as the standard medication for FC instead of PEG[6,37].

Stimulant laxatives: Stimulant laxatives, such as senna and bisacodyl, increase intestinal motility and interfere with water and electrolyte transport across the epithelial layer. Therefore, stimulant laxatives might result in cramping and abdominal discomfort[1,6,74]. When osmotic laxatives alone are ineffective in treating chronic constipation, stimulant laxatives are often considered. Although stimulant laxatives are thought to be safe and beneficial for treating childhood constipation, limited high-quality RCTs have evaluated their use[66,74,75]. Based on expert opinion, the use of stimulant laxatives may be considered as an additional or second-line treatment[37].

Lubricants: The most popular lubricant laxative is mineral oil, often known as liquid paraffin. Mineral oil works by coating and lubricating stools, lowering fecal water absorption in the colon, and making it easier to pass feces. Given that mineral oil has no chemical activity, severe negative impacts are rarely common. The effectiveness of mineral oil and oral laxatives in treating childhood constipation has been compared in a few low-quality trials. Accordingly, mineral oil promoted significantly greater bowel

movement frequency compared to lactulose[66,76,77], but no significant difference in treatment response was observed when compared to PEG[78]. Moreover, liquid paraffin was found to induce significantly better defecation frequency and FI episodes compared to senna; however, the evidence was of low quality[79]. Given the risk of aspiration and severe lipoid pneumonitis, mineral oil is not recommended for infants under the age of 1 year[80].

Promising pharmacological therapies

Probiotics/prebiotics: Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Probiotics have been used in the treatment of FC based on the hypothesis that they alter the intestinal microbiota and colonic pH, thereby improving gastrointestinal motility. Studies reported that constipated children have higher amounts of *Lactobacillus spp.*[81,82] and lower amounts of *Bacteroides*[83] compared to healthy children, implying that gut dysbiosis in the pathogenesis of constipation. So far, however, strong evidence to support the benefits of probiotics in the treatment or prevention of constipation has been limited[84-87]. However, some studies have demonstrated significantly increased stool frequency or softer stools after receiving probiotics. These findings might imply the significant impact of the pathogenesis of stool withholding in constipated children and that probiotics cannot be expected to overcome withholding behaviors. Therefore, future well-designed studies are needed.

5-HT$_4$ receptor agonists: Serotonin controls gut motility, visceral sensitivity, and intestinal secretion through serotonin 5-HT$_4$ receptors, which are primarily expressed by enteric nervous system interneurons[6].

5-HT$_4$ receptor agonists cisapride and tegaserod, which showed similar benefits for treating childhood constipation, were discontinued due to their increased risk for cardiovascular accidents and prolonged QT interval. Prucalopride is a new generation of selective, high-affinity 5-HT$_4$ receptor agonists that stimulate gastrointestinal motility and act primarily on parts of the lower gastrointestinal tract[6,74,88]. Current research on the benefit of prucalopride for constipated children has been contradictory. In an open-label pilot study, prucalopride had favorable effects on stool frequency, stool consistency, and frequency of FI in children with FC[89]. However, a recent multicenter RCT in 213 constipated children found no significant improvement of symptoms compared to placebo[90]. Common side effects include headache, nausea, abdominal pain, and diarrhea.

Chloride channel activators: Lubiprostone is a prostaglandin E$_1$ derivative that activates the chloride channel, thereby stimulating intestinal fluid secretion without increasing serum electrolyte levels[6,74,88]. A study on 127 children (3-17 years old) with constipation showed that lubiprostone was effective and well tolerated, with only minimal side effects like nausea and vomiting[91]. Another study evaluating the safety and tolerability of oral lubiprostone over the course of 24 wk for the treatment of childhood FC in patients aged 6-17 years old showed that lubiprostone was well tolerated and the frequency of treatment-emergent adverse events was similar to that seen in previous clinical trials and adults[92]. However, a double-blind, placebo-controlled, multicenter study on 606 children aged 6-17 years old (202 placebo; 404 Lubiprostone) with FC who satisfied the Rome IV criteria showed no significant difference in the total spontaneous bowel movements response rate between the lubiprostone and placebo groups. Frequently reported side effects include nausea, vomiting, diarrhea, and stomach pain[93].

Linaclotide and plecanatide bind to and act as an agonist of guanylate cyclase-C receptors, causing an increase in the production of chloride and bicarbonate the intestinal lumen. This increase in intestinal fluid causes an acceleration of the gastrointestinal transit while simultaneously decreasing visceral pain by reducing pain sensation[6,74]. Although no pediatric trials have been conducted, studies in adults showed that plecanatide treatment significantly improved constipation. The use of linaclotide in children with FC (0-18 years old) was only reported in a retrospective study, which showed that 45% patients with FC had a positive clinical response and approximately one-third of children experienced negative side effect, such as diarrhea, abdominal pain nausea, and bloating. Eventually, 27% patients stopped using linaclotid due to adverse events[94].

Herbal and traditional medicine: Stool withholding behavior is the main pathogenesis of FC in children. Given that successful toilet training takes time, normally more than 3-6 mo, osmotic laxatives have been the mainstay of treatment during toilet training. However, the majority of guardians and children wanted to withdraw the medication due to their concerns, sometimes with the taste or the amount of osmotic laxatives, which worsened the constipation. In several countries such as Iran, China, Vietnam, and Thailand, herbal medicine is usually integrated into some parts of the treatment as the main or additional therapy. Furthermore, while developing a new drug is a time-consuming and costly process, traditional drugs can be used to treat FC in children instead.

Several herbal and traditional medicines have been used for managing constipation in children, such as glucomannan[95,96], cocoa husk[97], AFPFF (combined acacia fiber, psyllium and fructose)[98], cassia fistula emulsion[99], inulin[100], black stap molasses[101], XiaojiDaozhi Decoction[102], damask roses[103,104], and other herbal medicines[105]. However, only a small number of herbal remedies for

FC have been well supported by RCTs in children (Table 6).

Most studies have reported that herbal and traditional medicine had significant effects on childhood constipation without significant adverse effects. Nonetheless, more high-quality studies are needed. This also suggests the need for studies that identify the active ingredients of herbal and traditional medicine responsible for their beneficial effects against FC in children. With more evidence, herbal or traditional medicine therapies can be integrated into standard treatments for childhood.

Nonpharmacological management

Diet (fiber and water): A low dietary fiber intake has been considered a risk factor for the development of FC[1,6,106]. The recommended adequate dietary fiber intake in children older than 2 years of age is equivalent to age (in years) plus 5-10 g/d[1,107]. However, there is still insufficient evidence from RCTs to support the routine use of fiber supplements to reduce constipation in children[108]. While some studies have shown that constipated children have lower fiber intake compared to healthy controls[96,109], other studies do not support this[95,110]. Regarding to fluid intake, lower water intake has been associated with a higher risk for intestinal constipation[111-113]. Hence, adequate water intake may be beneficial for the prevention of FC. The recommended water intake for children is based on the National Institute for Health and Care Excellence Guideline (Table 7).

Physiotherapy: FC in children is thought to be influenced by dysynergic defecation, which refers to pelvic floor dysfunction[88]. Studies have shown the effectiveness of pelvic muscle exercises as part of a combined treatment intervention[59,88]. A multicenter RCT comparing standard medical care (SMC), which includes education, toilet training, and laxatives, with pelvic physiotherapy (PPT) + SMC in constipated children aged 5-16 years old showed that PPT and PPT + SMC were effective in 63% and 92.3% of the children, respectively. Treatment success (based on the global perceived effect) were achieved in 88.5% and 33.3% of subjects receiving PPT + SMC and SMC, respectively ($P < 0.001$)[114]. A significant study in a primary care environment, however, found no additional benefits of PPT in 134 children aged 4-18[115] and that adding physiotherapy to SMA as a first-line treatment for all children with FC offered no cost benefits compared to SMA alone[116].

Toilet training: Toilet training aims to reduce symptoms, the child's defecation anxiety, and toileting avoidance[1,6]. There are four main methods for toilet training, including the child-oriented toilet training method, Azrin and Foxx method, Dr. Spock's toilet training method, and early elimination toilet training method[117-119]. The most friendly, accepted, and practical methods recommended by the American Academy of Pediatrics and Canadian Pediatric Society is the child-oriented toilet training method, in which the proper age for toilet training is between 18 and 24 mo[120,121]. Parents should be encouraged to be positive and supportive throughout the toilet training. Children should be encouraged to participate in toilet training, which consists of five sequential steps: know, dare, can, will, and do [122]. The child is taught to sit on the toilet for up to 5 min, one to three times a day, following meals to take advantage of the gastrocolic reflex[6]. The position of defecation is necessary to open the anorectal angle (the angle between the longitudinal axis of anal canal and the posterior rectal line, parallel to the longitudinal axis of rectum) and facilitate stool expulsion (Figure 10). To track improvement and compliance, keeping a daily journal of bowel movement, fecal and urine incontinence, and medication is beneficial. Providing stickers or small gifts as positive reinforcement for good behavior might further motivate children[6,122]. Through this process, children gain the ability to perceive their urge to defecate, consequently developing the habit of using the toilet instead of holding it in[59].

Biofeedback: Biofeedback training is a technique for teaching children how to control their perianal muscles for more efficient bowel movements. This technique involves bringing a typically unfamiliar physiological process to the patient's attention and allowing them to measure it[1,6]. In line with this, a recent meta-analysis incorporated three studies contrasting conventional treatment with add-on biofeedback treatment. Accordingly, two studies showed that treatment success rates were higher in the biofeedback group, whereas one study found no difference. In addition, one study found that the addition of biofeedback training at home offered no benefit in terms of defecation frequency compared to biofeedback at the laboratory[106]. Accordingly, biofeedback therapy is not advised for the regular treatment of children with FC based on the most recent research[59,106].

Abdominal massage: The mechanisms by which abdominal massages reduce constipation are most likely a combination of local stimulation and relaxation, as well as stimulation of the parasympathetic nervous system. Direct pressure over the abdominal wall alternately compresses and releases sections of the digestive tract, briefly distorting the lumen size and activating stretch receptors that can reinforce the gastrocolic reflex and trigger intestinal and rectal contraction[106,123,124]. A meta-analysis including a total of 23 RCTs and 2005 children showed that traditional Chinese medicine (TCM) infant massage had a superior effect on infant FC than drug therapy alone. Moreover, a clinical investigation found that children with FC may defecate more frequently and experience less constipation symptoms when receiving TCM infant massage[123]. There is little evidence to support the idea that using Chinese herbs in combination with other therapies might be beneficial[106,123]. Abdominal massage might be a promising additional therapy to manage FC.

Table 6 Characteristics of randomized controlled trials assessing the effects of herbal medications in children with constipation

Ref.	Country	Age (yr)	Study design	No. case (intervention/ control)	Intervention protocol	Probably pharmacological effect of herbal medicine	Duration of treatment/follow-up/end point and outcome measurement	Treatment effect
Esmaeilidooki *et al*[99], 2016	Iran	2-15	Open label, RCT, single center	109 (52/57)	CFE 1 mL/kg per day in three-divided doses (equivalent to 0.1 g of dried pulp of fruits of Cassia fistula). PEG 0.7-0.8g/kg per day	Phenolic antioxidants such as flavonoids, flavan-3-ol derivatives and anthraquinones: Stimulant laxative	Treatment for 4 wk. Primary outcome: Frequencies of defecation, severity of pain, consistency of stool, fecal incontinence and retentive posturing. Secondary: The safety and compliance of therapy	After 4 wk: 86.5% of children in CFE group and 77.1% in PEG group exited from the criteria of FC (RR = 1.121, CI95%: 0.939-1.338). Frequency of defecation that in CFE group (10.96 ± 5.7 stools per week) was significantly more than PEG group (6.9 ± 3.5 stools per week) (P < 0.001). No serious adverse effects in both groups (25% diarrhea and 3.8% abdominal pain)
Cai *et al*[161], 2018	China	1-14	Double-bline RCT, multicenter	480 (120/360)	XEBT: (1) 1-3 years old: 2.5 g, 3 times a day; (2) 4-6 years old: 5 g, 2 times a day; and (3) > 7 years old: 5 g, 3 times a day. Placebo	Seven herbs (Houpo contains magnolol, JueMingZi contain anthraquinones, LuHui contains reactive Aloe-emodin, BaiZhu contains Atractylodes japonica, LaiFuZi, XingRen, ZhiQiao): Promote small bowel peristalsis and work against atropine-induced small intestine suppression in mice	Treatment for 14 daysPrimary outcome: Frequency of SBM for 14 d. Secondary outcomes: Effectual time of defecation, mean symptom scores, disappearance rate of symptoms, recurrence rate and safety outcomes	The mean value of SBM for 14 d were 8.89 and 5.63 in the XEBT and placebo group (P < 0.05). The median effectual time of defecation, main symptom score and disappearance rate of symptoms were significant improved in XEBT group without the significant minor adverse effects between groups
Dehghani *et al* [101], 2019	Iran	4-12	Double-blind RCT, single center	92 (45/47)	BSM (sugarcane extract) 1 mL/kg per day. PEG 1 g/kg per day	The BSM naturally contained polyphenols (960 μg/mL), potassium (12430 μg/mL), iron (10 μg/mL), calcium (3320 μg/mL), zinc (22 μg/mL), sucrose (296000 μg/mL), triterpenoids (11230 μg/mL), phytosterols (7 μg/mL), flavonoids (2 μg/mL and polysaccharides (1250 μg/mL): Polysaccharide act as bulk forming agent, flavonoids/phytosterols and polyphenolic compounds act as natural antioxidants and anti-inflammatory agents	Treatment for 4 wk: Primary outcome: Response rate improvement in frequency of defecations per week, absence of lumpy or hard stools, abdominal pain and retention, soiling and blood-stained stool, sensation of anorectal obstruction/blockage. Secondary outcome: Patients' body weigh was measured in every visit and serological parameters (count blood cells, BUN, creatinine, calcium, phosphorus, sodium and potassium)	Defecation per week was significantly improved in both groups. Symptoms including volitional stool retention, large diameter stool, painful or hard stool and large fecal mass in the rectum decreased significantly two and four weeks after intervention (P < 0.05). No significant difference between the groups. No adverse effects were observed
Qiao *et al*[102], 2021	China	4-14	Double-blind RCT, multicenter study	200 (100/100)	Mixture of 12 herbs[1] (XiaojiDaozhi Decoction) and placebo (5% drug ingredients and 95% dextrin). All received fiber 20 g per day and toilet training)	12.5% *Raphanus sativus L.* (facilitating intestinal motility), 8.33% *Areca catechu L.* (stimulating gastrointestinal cholinergic receptor), 6.67% *Fructus aurantll immaturus* (stimulate gastrointestinal smooth muscle), *Citrus aurantium L.* (stimulate gastrointestinal smooth muscle), 6.67% *Crataegus pinnatifida* (stimulate gastrointestinal smooth muscle), *Magnolia officinalis Rehd* (stimulate gastrointestinal smooth muscle), *Cannabis sativa L.* (moistening the bowel and purging the stools), *Atractylodesmacrocephala Koidz.*	Treatment for 8 wk and follow-up for 12 wk. Primary outcome: Complete SBM (≥ 3 per week) and satisfaction with bowel function. Secondary outcome: Safety and adverse effect (blood measurement of liver and kidney function and lead level)	After 8 wk: 56% of CHM group and 25% of placebo satified with bowel movement (P < 0.05). 40% of CHM group and 19% of placebo had complete spontaneous bowel movement (P < 0.05). No serious adverse effects in both groups

					(stimulate gastrointestinal smooth muscle), *Semen armeniacae amarum* (purgative effect), *Paeonia lactiflora Pall*, *Radix et rhizoma rhei*, (purgative stool)0 and honey (moistening the bowel and purgative effect)		
Tavassoli *et al* [162], 2021	Iran	4-10	Open label RCT, single center	133 (66/67)	Viola flower syrups 5 mL, 3 times a day. PEG 4000 1 g/kg per day	Viola flower contains crude methanolic extract, butanolic and aqueous extracts the stimulate gastrointestinal motility	Treatment 4 wk. Primary outcome: Response off treatment (ROME III criteria). Secondary outcome: Stool consistency, defecation frequency, hard stools, painful defecation, fecal retention, and fecal soiling	Both groups demonstrated significant improvement in stool consistency, number off defecation, hard stool, painful defecation, fecal retention and fecal soiling at the end of the study compared to baseline ($P < 0.001$). No significant difference was observed between the two groups at baseline or at the end of the study ($P > 0.05$)
Nasri *et al* [163], 2021	Iran	2-15	Open-lable RCT	120 (60/60)	LaxaPlus Barij Syrup 1 mL/kg divided into 3 doses. PEG 0.7 g/kg	NA	Treatment and follow-up 8 wk. Primary outcome: Stool consistency, number of defecations, intensity of pain, fecal incontinence. Secondary outcome: Satisfaction rate	After 8 wk follow-up: Bowel movements in the intervention group was significantly higher than in the control group ($P < 0.05$). Pain intensity, and abdominal pain in the group LaxaPlus Barij® decreased significant than control group. No different about satisfaction rate between 2 group
Saneian *et al* [103], 2021	Iran	2-15	Double-blind RCT	60 (30/30)	Goleghand (including honey and Rosa damascene) 0.5 g/kg in three divided dose. PEG: 0.7 g/kg	NA	Treatment and follow-up 8 wk. Primary outcome: The number and consistency of stools per day, painful defecation, abdominal pain, and fecal incontinence. Secondary outcome: Adverse effects and parental satisfaction	After 8 wk: The number of fecal defecations in Goleghand group was higher than PEG ($P < 0.05$). The decrease of defecations after following was more significant in the PEG group than in the Goleghand® group ($P = 0.001$). Parental satisfaction scores did not change in either group ($P > 0.05$)
Imanieh *et al* [104], 2022	Iran	1-18	Double-blind RCT	100 (50/50)	Rosa damascena + brown sugar 1-2 mL/kg (1 mL composed of 0.1 g damask rose and 0.85 g brown sugar). PEG 1-2 mL/kg. All received high fiber diet and hydration	Damask rose: Osmotic laxatives and prokinetic effect. Brown sugar: Osmotic laxatives effect. Possible active ingredients might be phenolic compounds and aqueous fraction (terpenes, glucosides, flavonoids, anthocyanins, kaempferol and quercetin)	Treatment and follow-up 4 wk. Primary outcome: the effective of herbs with PEG. Secondary outcome: Adverse effects	After 4 wk: The cure rate was 100% in the R. damascena group and 91.7% in the control group. Adverse effect of intervention group was the taste which was too sweet

[1]LaxaPlus Barij® Syrup- an herbal medicine that includes *jujube, rose, asparagus, violet flower, borage, quince seeds, and Cordia myxa fruit*. CFE: Cassia fistula emulsion; PEG: Polyethylene glycol; FC: Functional constipation; RR: Risk ratio; RCT: Randomized controlled trial; XEBT: Xiao'er Biantong granules; SBM: Spontaneous bowel movements; BSM: Black strap molasses; BUN: Blood urea nitrogen; CHM: Chinese herbal medicine.

Retrograde enemas: There is insufficient evidence to support the role of retrograde enemas in the maintenance phase of FC in children. Hence, this procedure is reserved for intractable constipation, especially in cases with slow-transit constipation or megarectum. An RCT comparing to the clinical efficacy of supplemental treatment with rectal enemas against conventional treatment alone in 100 children between the ages of 8 and 18 who had symptoms of constipation for at least 2 years revealed that defecation frequency normalized after 1 year of treatment in both groups but was significantly

higher in the intervention group compared to controls at 26 and 52 wk (5.6/wk *vs* 3.9/wk, $P = 0.02$, and 5.3/wk *vs*. 3.9/wk, $P = 0.02$, respectively). Enemas as maintenance therapy for severely constipated children had no substantial side effects compared to oral laxatives alone[125].

Transanal irrigation: Transanal irrigation (Figure 11A) can be an option for children with FC who do not respond to pharmacological treatment[126,127]. It involves inserting a catheter or cone into the rectum to inject water into the colon, cleaning it completely[126]. This treatment has been previously well established for patients with neurogenic bowel disorders and anorectal malformations[127]. Moreover, evidence has suggested its safety and effectiveness, with an average success rate of 78% for both FI and constipation[127,128]. In addition, 86% of the parents were satisfied with the results of transanal irrigation, and 67% reported that they would continue using transanal irrigation for the treatment of their child's symptoms[129]. Therefore, transanal irrigation can be considered an effective therapeutic option for severely constipated children with FI who do not respond to conventional therapy[6,127,128].

Botulinum toxin A injection: Botulinum toxin A (Botox) injections into the anal sphincter can be considered in cases suspected of anorectal dysfunction or functional outlet obstruction[59]. Botox injections, which temporarily reduce anal sphincter muscle contraction, serves as both a diagnostic test, indicating whether the obstructive symptoms are being caused by internal anal sphincter hypertonia, and treatment for intractable constipation. Children who exhibit significant withholding behavior or anal sphincter dysfunction may benefit from Botox injections[6,59]. A RCT and systematic review found that Botox injections were as equally effective as internal sphincter myectomy on short-term follow-up [130,131]. However, a retrospective study on 164 children over 7 years old with intractable constipation showed that Botox injections into the internal anal sphincter of children had an overall response rate of 70%. Moreover, anorectal manometry studies in children with normal and abnormal sphincter dynamics observed similar response rates to this therapy[132].

Neuromodulation: Sacral neuromodulation (SNM) is a promising option for the treatment intractable constipation. SNM involves percutaneous placement of an electrode into the third sacral foramen and implantation of a stimulating device under the skin covering the buttocks[6]. The exact working mechanism of SNM remains largely unknown, although evidence has suggested that SNM stimulates anorectal function at a more central level. SNS can affect multiple physiological functions of the pelvis and lower abdomen and supports the propulsive peristalsis of the intestine, which is of special interest in slow-transit constipation[133]. One study in 30 constipated children reported a significant improvement in defecation frequency and abdominal pain after 3 wk of SNM treatment, with the effects being sustained over 22 mo of follow-up in 42% of children. Another study on the treatment of intractable constipation with SNM for over 2 years found that defecation frequency did not change after SNS; however, patients reported that FI decreased from 72% to 20% ($P < 0.01$) and urinary incontinence decreased from 56% to 28% ($P = 0.04$). Minor complications include pain after implantation, displacements of the leads, and infection[134,135]. A recent pilot study that assessed noninvasive SNS in 17 constipated children also found it effective in improving symptoms of constipation[136]. Additionally, abdominal transcutaneous electrical stimulation and posterior tibial nerve stimulation, two skin stimulation techniques, have been used to neuromodulate the bowel to treat constipation with promising outcomes[6].

Surgery: Although almost all patients with FC are successfully treated with conventional therapy, a few continue to have intractable symptoms without any organic problems. In such cases, surgical interventions may be beneficial.

Apart from transanal irrigation, there are also surgical treatment options to achieve stool expulsion, such as antegrade colonic irrigation or antegrade continence enemas (ACEs)[59]. When maximal conventional therapy fails, ACEs have been considered a successful therapeutic option for constipated children[1,6]. ACEs allow fluid to be flushed through the entire colon *via* an external opening into the colonic lumen, which is usually located at the cecum. The most well-established ACE procedures are percutaneous cecostomy and Malone appendicocecostomy (Figures 11B and C). The success rate of ACEs for the management of FC varies from 15% to 100% among studies[130,137,138]. Various enema solutions can be used, including saline and PEG. Complications include skin abrasion, stoma stenosis, granulation tissue, enema fluid leaks, and tube dislodging[137,138]. Regarding other surgical management, there are no clear guidelines on surgical colonic resections and ostomies for children with FC, with such surgical management only reserved for severely constipated children who do not respond to conventional therapy and surgical ACE. These surgical procedures are performed in specialized centers by a multidisciplinary team due to their complexity and potential for problems[6,59].

Table 7 Recommend water intake per day[164]

	Total water intake per day, including water contained in food (mL)
Infants 0-6 mo	700 (water is assumed to be from breast) milk
7-12 mo	800 (milk and complementary foods and beverages)
1-3 yr	1300
4-8 yr	1700
Boys 9-13 yr	2400
Girls 9-13 yr	2100
Boys 14-18 yr	3300
Girls 14-18 yr	2300

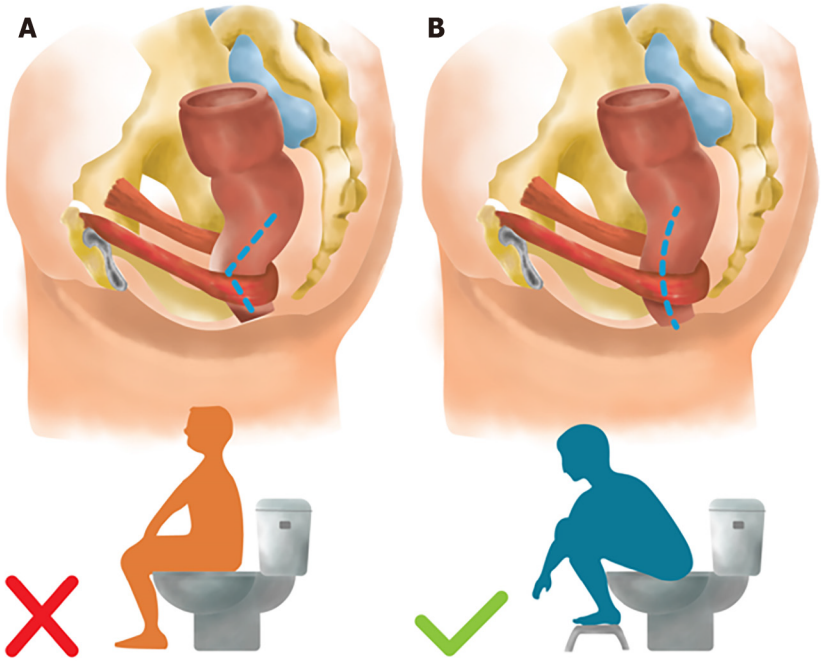

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Figure 10 Position during defecation. A: Improper position during defecation; B: Proper position to open the rectoanal angle and facilitate stool expulsion during defecation.

CONCLUSION

FC is the most common FGID in children and it affects the quality of life and psychological health of both the child and the family. Stool withholding behavior is the main etiological agent of FC and successful toilet training is the most effective treatment measure as it also prevents FC recurrence in the long term. Nonetheless, osmotic laxatives and lifestyle modifications, along with adequate fiber and fluid intake, are also crucial as first line therapy during toilet training. Even though extensive history taking and physical examination might enable a diagnosis of FC according to Rome IV criteria, children with intractable constipation may require multiple investigations to confirm the diagnosis or to exclude organic causes. Apart from osmotic laxatives, other promising herbal and alternative therapies have been reported to yield satisfactory outcomes in FC with minimal short-term adverse effects; nevertheless, more evidence is needed before these strategies can be adopted worldwide. Children with intractable constipation typically requires a multidisciplinary team approach and the physician should refer the child to a pediatric specialist for re-evaluation and further management (Figure 12).

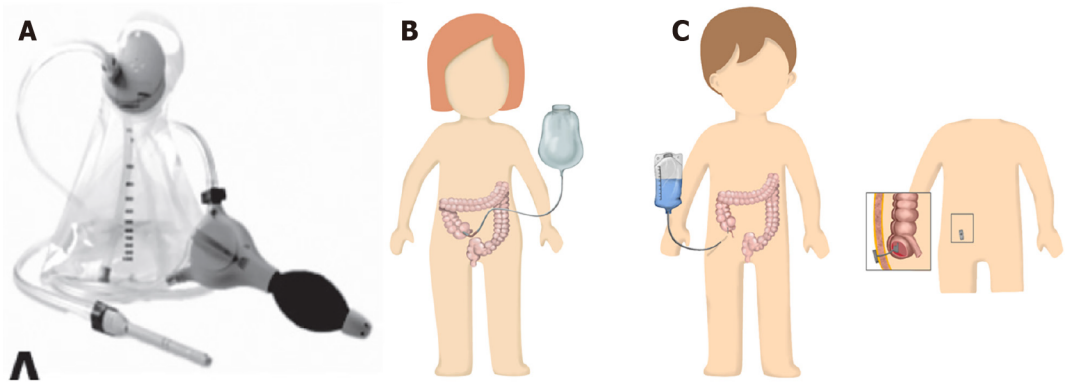

Figure 11 Rectal irrigation method. A: Transanal irrigation; B and C: Antegrade continence enema. A-C: Citation: Palittiya Sintusek. Pediatric lower gastrointestinal motility disorders. In: Werawatganon D, editor. Practical Neurogastroenterology and motility: Basic, testing, and treatment. Bangkok: Printable Inc, 2021: 423-424. Copyright ©The Authors 2021. Published by Thai Neurogastroenterology and Motility Society. The authors have obtained the permission for figure using from Thai Neurogastroenterology and Motility Society (Supplementary material).

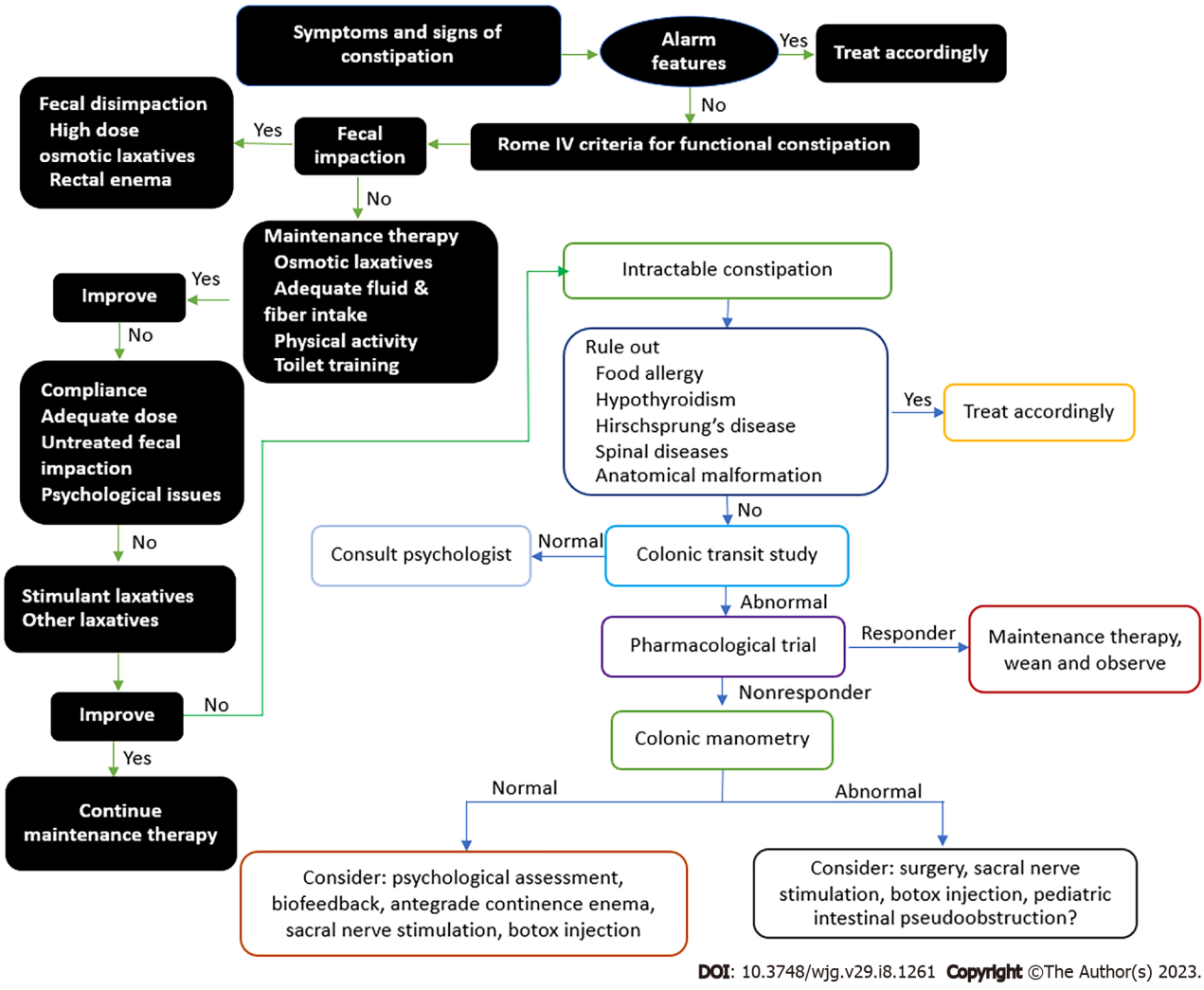

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Figure 12 Algorithm for the management of children presenting with signs and symptoms of constipation.

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FOOTNOTES

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Country/Territory of origin: Thailand

ORCID number: Duc Long Tran 0000-0002-2576-9209; Palittiya Sintusek 0000-0003-4441-0151.

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REVIEW

Early-onset colorectal cancer: A review of current knowledge

Margarida R Saraiva, Isadora Rosa, Isabel Claro

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Margarida R Saraiva, Isadora Rosa, Isabel Claro, Department of Gastroenterology, Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa 1099-023, Portugal

Corresponding author: Isadora Rosa, PhD, Medical Assistant, Department of Gastroenterology, Instituto Português de Oncologia de Lisboa Francisco Gentil, Rua Prof Lima Bastos, Lisboa 1099-023, Portugal. isarosa@ipolisboa.min-saude.pt

Abstract

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide. Although most prevalent among older people, its incidence above 50 years old has been decreasing globally in the last decades, probably as a result of better screening. Paradoxically, its incidence in patients below 50 years old [early-onset CRC (EO-CRC)] has been increasing, for reasons not yet fully understood. EO-CRC's increasing incidence is genre independent but shows racial disparities and has been described to occur worldwide. It follows a birth-cohort effect which probably reflects a change in exposure to CRC risk factors. Its incidence is predicted to double until 2030, which makes EO-CRC a serious public health issue. Both modifiable and non-modifiable risk factors have been identified - some are potential targets for preventive measures. EO-CRC is often diagnosed at advanced stages and histological features associated with poor prognosis have been described. EO-CRC presents some distinctive features: Microsatellite instability is common, but another subtype of tumours, both microsatellite and chromosome stable also seems relevant. There are no age-specific treatment protocols and studies on EO-CRC survival rates have shown conflicting data. Due to the higher germline pathological mutations found in EO-CRC patients, an accurate genetic risk evaluation should be performed. In this review, we summarize the current evidence on epidemiological, clinical, histopathological and molecular features of EO-CRC and discuss the contribution of genetics and lifestyle risk factors. We further comment on screening strategies and specific dimensions to consider when dealing with a younger cancer patient.

Key Words: Colorectal cancer; Early-onset; Adenocarcinoma; Hereditary; Birth-cohort effect; Risk factors

Core Tip: The incidence of early-onset colorectal cancer (EO-CRC) has been surprisingly increasing worldwide and it has become a public health issue. Its clinical, genetic, molecular and histological characteristics suggest that this may be a distinct entity, with a more aggressive behaviour. However, both genetic and environmental risk factors seem to contribute to this observed epidemiological shift in CRC incidence. More evidence is needed in order to clarify EO-CRC aetiology and to develop screening and management strategies.

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INTRODUCTION

Colorectal cancer (CRC), the most common gastrointestinal cancer, is the third most common malignancy and the third leading cause of cancer-related death globally[1]. Once perceived as an elderlies' disease, CRC incidence among the younger population [early-onset CRC (EO-CRC)] has been gaining more relevance. Recent epidemiological data show that, probably due to interventions on risk factors, screening programs implementation and better treatment[2], the incidence and mortality of CRC in patients older than 50 have been decreasing for the past decades[3]. On the contrary, from 1939, when the first case-reports of CRC in the young were published[4], the incidence of EO-CRC has been increasing and is expected to double by 2030[5].

Efforts have been made to characterize EO-CRC, questioning if this is a different entity from CRC in the older. As consensus is lacking, the authors review the existing knowledge, namely epidemiology, risk factors, clinical, molecular and genetic characteristics as well as treatment and overall survival (OS). EO-CRC has become a global health issue and there is a need to raise awareness for it, aiming at lowering the suspicion diagnostic threshold for symptomatic young patients.

EPIDEMIOLOGY

CRC belongs to the top 3 of the most prevalent cancers worldwide, representing, in 2020, 10% of the global cancer incidence and 9.4% of all cancer-related deaths[1,6]. Globally, both CRC incidence and mortality have been decreasing in the last decades[3,5], which is consistently associated to screening programs implementation, thus allowing the detection and removal of adenomatous polyps and consequently interrupting the adenoma-carcinoma sequence[7]. In fact, the inflection time point when CRC incidence began to decrease occurred in the 1990s, coincident with the adoption and diffusion of colonoscopy for screening of average-risk CRC patients in several countries[8].

CRC incidence follows a heterogeneous geographical distribution. The highest incidences have been reported to occur in China, United States of America and Japan. Studies suggest that Human Development Index (HDI) is positively correlated with CRC incidence[9], meaning that countries with high HDI have a CRC incidence about 4-folds higher than low HDI countries. Currently, developed countries show a stabilizing or declining incidence rate whereas developing countries have an increasing incidence rate. The later is probably related to the increase in exposure to CRC risk factors[1], reflecting the adoption of a western lifestyle that accompanies economic transition[10].

Current data implies that CRC is going through an epidemiological change. In fact, based on data from the United States Surveillance, Epidemiology, and End Results program, Galloway *et al*[11] concluded that, although CRC incidence is decreasing by 3.1% per year among people above 50 years old, the incidence of EO-CRC is increasing by 1.4% per year. EO-CRC patients currently account for a percentage that ranges from 0.4% to as high as 35.6% with a median of 7% between the considered reports - such high values should be interpreted carefully as they may reflect institutional biases due to cancer centres that probably receive a high referral of EO-CRC patients[12]. Undoubtedly, CRC has become an increasingly more common diagnosis in younger patients.

One problem potentially leading to controversial research results is the lack of an agreed definition of EO-CRC patients. Some authors use a cut-off of 40 years old[12,13], but most reports in the literature apply a 50 years old limit[10,14,15], this later being consistent with the Amsterdam criteria, that identify patients more likely to have an hereditary predisposition for CRC[16]; 50 years old also coincides with the starting age of most screening programs in the general risk population.

Most studies found no significant difference in gender distribution in CRC[12,17] and the available evidence on EO-CRC prevalence found it to be independent of gender as well[13]. Racial disparities in EO-CRC incidence have however been described, namely with a higher proportion of Black patients,

who also have a worse OS rate[18] - the same occurs for CRC in general in the United States[19]. A recent study, that suggested that the EO-CRC incidence has increased in all racial groups, reported the most elevated rise in Non-Hispanic White People. However, the overall incidence of EO-CRC in the United States remains higher for African Americans[20]. In an Israeli study, racial differences were also found, with the highest EO-CRC incidence in the Arabic population[21].

The reasons for the increased incidence of EO-CRC are not fully understood. Some authors suggest that the common use of colonoscopy is responsible for a proportion of the detected CRCs in young adults. This may have happened as a consequence of the diffusion of colonoscopy as a screening method as well as of an increased endoscopic capacity and efficiency, which may have unintentionally lowered the threshold for performing colonoscopy in younger patients[22].

Another important factor to consider when reflecting about EO-CRC incidence is the globally described existence of a strong birth-cohort effect. This is considered to occur when age-specific incidence rates vary by generation as a result of changes in people's exposure to factors associated to disease risk. This is the opposite concept of period effect, when incidence varies at the same time for all generations[23].

A report based on the incidence of CRC in people from 20 European countries, aged 20 to 49 years old and including data from 1990 to 2016, elucidates on this effect. It showed that the CRC incidence for people aged 30-39 years old started to increase exactly 10 years earlier than in the 40-49 years old group and that, although the CRC incidence also increased for 20-29 years old patients, the study period (> 1980) did not cover the time inflection for that group. This suggests that the turning point possibly occurred before 1990. Additionally, the biggest incidence rise occurred in patients 20-39 years[24], which is consistent with results from an American study[5].

This recent rise in EO-CRC has been observed globally. A report including data from 20 European countries found that EO-CRC incidence has raised significantly in 14 of them, in 5 of them was stable and decreased only in Italy[24]. Another study about EO-CRC incidence among 36 countries from 5 continents reported that EO-CRC incidence was stable in 14 countries, decreased in 3 countries (Austria, Italy and Lithuania) and increased in 19 countries. From these 19 countries, 9 presented an isolated increase in EO-CRC incidence rate - in 5 of them CRC incidence was declining in the older population and in the remaining 4 countries it was stable[17].

Additionally, authors concluded that the most rapid increase in EO-CRC happened in countries where incidence rates were already the highest, such as South Korea[10]. This may be justified by the modernization process that occurred earlier in South Korea in comparison with other Asian countries and the consequently rapid dietary transition that took place following the Korean war[25]. The obesity increase that has been occurring worldwide, both for adults and children, may also play an important role, particularly because Asian countries have been experiencing some of the largest relative increases in body mass indexes[26].

In terms of future predictions, it is expected that the incidence rate for EO-CRC will double by 2030, while in older patients incidence it will probably decline by more than one-third. Meaning that, compared to 2010, when only 4.8% of all colon and 9.5% of all rectal cancers were diagnosed in younger patients, by 2030 they will sum up to 10.9% of all colon and 22.9% of all rectal cancers[5]. These findings explain why EO-CRC is clearly becoming an important public health issue.

RISK FACTORS

CRC is a very heterogeneous disease that results from an interaction between genetic and environmental factors. The majority of CRCs are sporadic (70%), a small proportion of cases are caused by inherited syndromes (5%) and the remaining (25%) have an associated hereditary component, which has not yet been well established and is known as familial CRC[27]. Based on current knowledge, both modifiable and non-modifiable factors may also contribute to EO-CRC.

Modifiable risk factors

There is substantial controversy about results from studies concerning risk factors for CRC, and for EO-CRC in particular, especially concerning preventable risk factors. The majority of data about CRC risk factors is based on older age cohorts' evidence and mainly represents mid- to late-life exposures. But when it comes to EO-CRC, early-life exposures may have a predominant role[28].

One of the most consensual risk factors for CRC is excess body weight. This has been associated with an increased risk for CRC[29] and has become more relevant since obesity prevalence has increased in the last decades[30], paralleling the tendency of EO-CRC[31]. In fact, recent data show that obesity is responsible for a 20% higher risk of EO-CRC[32].

Early-age exposures and weight are also suggested to influence CRC risk. A meta-analysis concluded that higher body fatness at an early age is associated with a higher risk of colon cancer in men and women, the same not applying to rectal cancer[33]. This correlation is probably explained by the effect of proinflammatory cytokines produced by the adipose tissue and by the chronic exposure to hyperinsulinemia and insulin-like growth factor 1 (IGF-I), that may contribute to carcinogenesis[34].

Another consistently associated risk factor for CRC is diabetes mellitus, with an increased relative risk of 30%[35]. In fact, a Swedish study concluded that a diabetic patient reaches the 10-year cumulative risk for CRC that justifies the general screening program 5 years earlier than a non-diabetic patient[36]. Its impact on EO-CRC has already been proved as well[37].

Both alcohol[38] and tobacco[39] are independent risk factors for CRC. A recent study also showed their contribution to EO-CRC, particularly when concomitantly present[40,41]. The protective role of physical activity has been described in different studies that demonstrated an inverse relationship between physical activity and risk of colon cancer. This probably happens due to decreased inflammation, reduced intestinal transit time, decreased IGF-I levels, reduced hyperinsulinemia and modulated immune function, that come with physical activity[42].

Conflicting data exists regarding the role of dyslipidaemia in CRC risk. While some studies suggest a protective one[43], others associate it to a higher risk[44]. The association between diet and CRC has been extensively studied. Some of the proposed elements associated to a higher risk of CRC are processed and red meat[45-47] and sugar sweetened beverages[48] - these are believed to influence gut immune response and inflammation[49]. On the contrary, a diet rich in fiber, dairy, fruits, vegetables [45], fish, beta-carotene, vitamin C, vitamin E, vitamin D and folate has shown a protective effect[41,50].

Aspirin and nonsteroidal anti-inflammatory drugs also exert a dose-dependent protective effect for CRC, through inhibition of the cyclooxygenase pathway[51]. Some of these interactions may account for the older onset/EO-CRC findings[41]. The importance of including fiber in the diet is mainly due to the production of short chain fatty acids during fiber fermentation. These have anti-inflammatory and anti-tumor properties[52] and their levels are inversely related with CRC occurrence[53].

The Western diet, typically rich in red and processed meat and poor in fiber, may be implicated on some of the differences found in incidence among populations[49]. Currently, the increasing incidence of EO-CRC is mostly observed in high-income economies or countries transitioning to a high-income economy and thus adopting Westernized lifestyle habits such as a western diet, weight excess and less activity level - all these may contribute to the increase in EO-CRC[10].

Microbiota has been recently proposed to also play a role in CRC pathogenesis. After comparing the colonic bacterial flora of populations considered to be at high and low risk for CRC[54], differences in microbiota composition were described - this may be a consequence of dietary habits and could harbour a risk for the development of CRC. Besides diet, the use of antibiotics may also affect the microbiota composition and it has been showed that the use of antibiotics, especially when used repeatedly[55] or for long periods during early to middle adulthood[56], is associated with an increased risk of developing CRC. From this point on, further research is needed before a microbiota modifying approach is considered. Current data shows conflicting results on prebiotic and probiotic therapy in CRC patients[57,58]. Based on the already mentioned birth-cohort effect, it seems that modifiable risk factors may play an important role in EO-CRC pathogenesis. The majority of the already known modifiable factors for CRC in general are believed to also apply to EO-CRC[41]. However, conflicting and limited evidence demands more studies.

Non modifiable risk factors

The most consensual risk factor for CRC is a positive family history[17]: First-degree relatives (FDR) have an increased risk of developing CRC, especially when the proband was diagnosed at a younger age. In fact, having a FDR diagnosed under the age of 50 increases the risk of developing CRC by more than three-fold[59]. This risk is also increased for people with more than one affected relative[59], and for family history among distant relatives[60]. Additionally, a history of advanced adenomas or even a family history of any kind of adenomas also increases CRC risk[61,62].

Other known conditions that increase the risk for EO-CRC are Lynch syndrome (LS) and familial adenomatous polyposis, among other hereditary CRC syndromes, with a larger proportion of EO-CRC being hereditary compared with older CRC patients[12,63] (Figure 1). Additional risk of EO-CRC has also been described for patients treated with pelvic radiation at young age[17,64].

Finally, patients with inflammatory bowel disease (IBD) are also known to be at increased risk for CRC[54], due to long-term inflammation. The highest risk is associated with extensive long duration colitis and/or a concomitant primary sclerosing cholangitis diagnosis. The association with EO-CRC, especially for ulcerative colitis, has been clearly demonstrated[15].

Conflicting data has been published about the correlation between breast cancer and CRC. Several studies have suggested an increased risk of CRC for patients with breast cancer at younger ages, and this relationship has also been suggested for EO-CRC[15].

CLINICAL AND HISTOPATHOLOGICAL CHARACTERISTICS

Evidence from different studies found some differences between EO-CRC and older patients CRC, concerning clinical presentation, location and histological characteristics, suggesting it might be a distinct entity. Most studies have agreed on the clinical features of EO-CRC, namely on the most common presentation symptoms, with abdominal pain, rectal bleeding, weight loss, anaemia, decreased

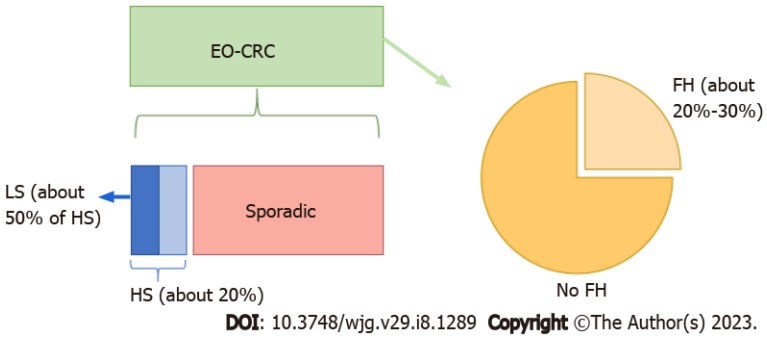

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Figure 1 Genetic risk in early-onset colorectal cancer. HS: Hereditary cancer syndromes; LS: Lynch syndrome; FH: Family history of colorectal cancer; EO-CRC: Early-onset colorectal cancer.

appetite and change in bowel habits[12,52,65], as well as on the more frequent presentation with bowel obstruction[66]. These common clinical symptoms are in accordance with the most common location of EO-CRC in the left colon, more precisely the rectum and the sigmoid colon, as described in the United States[12,23,67-69]. However, global data regarding location are conflicting.

A delay in the diagnosis of EO-CRC has been consistently described, with one series showing an average delay of 4[70] to 6 mo[71] and even with case-reports of a 2 years delay[72]. This represents a 1.4-fold increase in time when compared to older patients[70]. Various reasons can justify this, both doctor and patient related. There is probably both a lower suspicion by the clinicians, hence assuming that the symptomatology is associated to other benign and more common pathology (such as irritable bowel syndrome or haemorrhoids) and consequently not investigating further, and a younger patients' delay in seeking medical attention, out of fear, denial, absence of information about alert symptoms, difficult access to health care or financial reasons[17,72,73].

Several studies consistently demonstrated that younger patients present more often with stage III or IV disease[10,66,74]. In fact, one multicentre retrospective study found that 61.2% of EO-CRC compared to 44.5% of older patients presented metastatic disease at diagnosis[12,13,75]. A plausible explanation for this relates to the absence of screening programs for younger patients but it may also be due to aggressive histopathological characteristics of EO-CRC and to its potential genetic basis, that may predispose to accelerated carcinogenesis in young patients. The diagnostic delay in younger patients may also contribute to more advanced disease at EO-CRC diagnosis[76], although this is not completely explained by the longer time to diagnosis[70].

Furthermore, synchronous or metachronous tumours more frequently arise in EO-CRC[76] but precursor adenomatous lesion are less frequently identified in the EO-CRC groups compared with older ones[13,52] - this also favours the accelerated carcinogenesis hypothesis. Histological predictors of bad prognosis have also been often found in EO-CRC. Young patients' tumours more frequently display adverse histologic features compared with older CRC patients, such as mucinous or signet cell differentiation and poorly differentiated tumours[13,66,68,77]; lymphovascular, venous, and perineural invasion have also been described to be more common[13,66].

MOLECULAR AND GENETIC FEATURES

Since the classical sequence adenoma-carcinoma implicated in the carcinogenesis of CRC has been proposed, our understanding of its biology has evolved and currently CRC and particularly EO-CRC is believed to be an heterogenous disease, including cases with a strong hereditary component as well as sporadic disease[78]. For every CRC patient, a genetic aetiology should be considered, and this is particularly important for EO-CRC patients. In the EO-CRC group, even if we exclude LS (the most common hereditary CRC syndrome) patients, there is still a clear familial component, stronger than for older patients[78].

It is estimated that 20%-30% of CRC are associated with a family history of colorectal polyps or cancer, and that up to 3%-5% of CRC are attributed to an identifiable inherited CRC syndrome, such as LS, familial adenomatous polyposis, MUTYH-associated polyposis, juvenile polyposis syndrome, Peutz-Jeghers syndrome or others[79]. Recent studies concluded that 1 in 5 of EO-CRC cases is attributed to hereditary cancer syndromes[63], and half of those particularly to LS[80] (Figure 1).

Observing mutational profiles of left-sided CRCs, EO-CRC patients were found to have higher rates of mutations in genes related to cancer-predisposing syndromes, such as *MSH2* and *MSH6* (LS), neurofibromatosis type 1, *PTEN* hamartoma tumours syndrome or Cowden's syndrome, tuberous sclerosis complex (*TSC*)*1* and *TSC2*, and *BRCA2* (hereditary breast and ovarian cancer syndrome)[81].

Due to the fact that a larger proportion of EO-CRC seems to be hereditary compared with older patients CRCs, the diagnosis of EO-CRC is currently an indication for referral for genetic evaluation [82]. Additionally, the variability in clinical presentations and potential for phenotypic overlap in CRC justifies the recommendation for genetic testing using a multigene panel for all individuals with an age below 50 years old[83]. This next-generation genetic tests identify pathogenic germline variants in 16%-20% of EO-CRC cases, including genes with moderate to high penetrance in cancer syndromes[63,80].

The diagnosis of a hereditary cancer syndrome frequently influences therapeutical options. In fact, it determines the surgical approach (*e.g.*, subtotal colectomy instead of segmental resection), the chemotherapy regimen choice, colonoscopy surveillance intervals, and management of potential extracolonic cancers[84]. Furthermore, genetic testing is also important to identify relatives that may benefit from anticipation of colonoscopy screening[85].

Although germline genetic alterations can be implicated in 20% of individuals with EO-CRC, hereditary syndromes continue to account for only a minority of cases and the majority of EO-CRC are sporadic, in patients with no family history of CRC[86,87]. CRC represents a heterogenous group of cancers, although its majority can be divided in a few molecular subtypes, that influence treatment options such as chemotherapy regimens and correlate with cancer survival. Interestingly, current evidence suggests a different proportion of molecular tumour subtypes between older and EO-CRCs[68,78,88,89].

CRC usually follows one of the three main molecular subtypes: Chromosomal instability (CIN) or suppressor pathway subtype (80%-85%), CpG island methylator phenotype (CIMP) or serrated pathway (40%) or microsatellite instability (MSI) or mutator pathway subtype (10%-15%). The later is characterized by MSI due to loss of DNA mismatch repair (MMR), occurring both in a sporadic manner or in association with germline alterations in MMR genes (LS), as reviewed in Currais *et al*[84]. These subtypes are not mutually exclusive and can frequently overlap, with a minority of CRCs exhibiting both microsatellite and chromosome stability (MACS)[78,89].

The currently available studies addressing the molecular profile in EO-CRC have reached conflicting results. Some authors present evidence that EO-CRC tumours present more frequently with MSI (MSI-H)[81,90]. MSI tumours in these patients are mostly related to LS[91] and rarely to inactivation of MLH1 (which commonly occurs in older patients) but are more frequently associated with MSH2 inactivation [92]. They are associated with a better prognosis than microsatellite stable (MSS) tumours[93]. Other reports describe that the majority of EO-CRC are rather MSS, lacking DNA repair mechanism abnormalities[94,95]. EO-CRC MSS tumours are typically localized in the left colon, rarely associate with other primary neoplasms and have a strong familial component, features that distinguish them from MSS CRC in older patients[94].

MMR status has potential therapeutical implications. For example, CRCs exhibiting MMR deficiency typically have poor response to fluorouracil-based adjuvant chemotherapy[93]. Interestingly, however, patients with MMR-deficient tumours tend to exhibit better OS, attributed to the more immunogenic nature of these tumours[89]. Additionally, metastatic tumours with MMR deficiency are candidates for immune checkpoint inhibitors[88].

The CpG island methylator phenotype, seems to play a modest role in EO-CRC. EO-CRC are frequently CIMP-low except for EO-CRC LS patients, for whom a higher proportion will be CIMP-high, compared to those who develop LS-related CRC later in life[94]. A more recently described subset of CRC, defined by MACS, because of their diploid DNA content and lack of MMR deficiency, may account for 30% of all sporadic CRCs. These affect younger patients more commonly and have been identified frequently in the left distal colon and rectum. Moreover, this molecular subtype has been associated with poor differentiation, early occurrence of metastasis, disease recurrence and lower survival than patients with MSI or CIN. This can be related to its lack of immune response, opposite to the immunogenic favourable properties of MSI-H CRC[89]. There is still an incomplete understanding of the molecular profile of MACS. Silla *et al*[78] reviewed its main features: MACS tumours are usually CIMP-low, are rarely associated with *BRAF* mutations, lack MLH1 expression, and present a different pattern of hypomethylation than MSI and CIN CRC. Some published studies suggest that MACS may be related to familial CRC syndromes, based on observed increased frequency in young patients[96].

LINE-1 hypomethylation is another feature implied in EO-CRC and is considered to be a surrogate marker for genome-wide hypomethylation. It is associated with increased CIN[97]. Evidence shows that the degree of LINE-1 hypomethylation is an independent factor for increased cancer related mortality and overall mortality in CRC[98]. Antelo *et al*[91] compared this marker in EO-CRC and older CRC and concluded that the first group presented lower levels of LINE-1 methylation, which suggests a distinct molecular subtype of these tumours. Additional studies are needed in order to confirm this association and properly assess the prognostic value of LINE-1 in young-onset CRC.

As for the consensus molecular subtypes classification, EO-CRC tumours are more likely to have subtypes CMS1 or CMS2 tumours compared with CMS3 or CMS4 tumours[68]. From a mutational perspective, despite some conflicting data, the most consensual results show that EO-CRC has lower prevalence of *BRAF* V600, *NRAS*, *KRAS* and *APC* mutations in comparison with late-onset CRC[13,68].

In order to rigorously determine if the described characteristics are sufficient to consider EO-CRC as a distinct molecular profile, more and larger trials are needed. In fact, although lifestyle factors and exposure are believed to contribute to the EO-CRC incidence increase, germline and molecular data also

provide clues to a different CRC pathogenesis.

TREATMENT

EO-CRC does not have specific evidence-based treatment protocols. The subset of EO-CRC patients that present an inherited CRC syndrome, such as LS and polyposis syndromes, should follow their respective guidelines. This is important as the surgical approach is generally more radical and there is a need to manage the risk for other syndrome related malignancies by appropriate screening[3,99].

For the majority of the patients, although there are no age specific recommendations, current studies show that different therapeutic strategies are being implemented according to the patient's age. Surgery is the main curative treatment for CRC, but for high-risk stage II and stage III there is a role for adjuvant chemotherapy[100]. When comparing EO-CRC with an older cohort (65-75 years old), various studies have found that more patients with early-stage EO-CRC are treated with adjuvant therapy for stage II and III disease. In fact, stage II low-risk EO-CRC patients receive adjuvant therapy 50% of the time, compared with 19.1% in the older cohort[101,102]. Curiously, these regimens were not associated with increased survival in stage I or stage II cancers and offered only marginal benefit in stage III and stage IV diseases[102].

Additionally, EO-CRC patients have better chances of receiving surgical treatment either for early-stage or for metastatic disease[66,103], of receiving radiotherapy at all disease stages and also of being offered more aggressive adjuvant treatment, including multi-agent chemotherapy[102]. Another study found that EO-CRC patients with stage I or II were more likely to receive adjuvant or neoadjuvant therapy, compared to older patients[66].

These studies may reflect the fewer comorbidities of EO-CRC patients, a better performance status and fewer adverse reactions to systemic treatment leading to a better tolerance to multiagent regimens than the older patients[66]. However, they may also show a bias towards offering more treatment to younger patients and there is a question of whether they are being victims of overtreatment with no proven benefit. According to current knowledge, equal treatment should be offered to EO-CRC (without a hereditary syndrome). More studies on the putative molecular differences between EO-CRC and older age CRC may in the future bring new molecular treatment targets, allowing specific treatment[11].

SURVIVAL

Some authors, supported by population-based studies, suggest that EO-CRC presents a better survival at every stage[77,103] and even that 5 years after curative treatment, EO-CRC survivors present the same main causes of death than the general population[104]. Other studies demonstrated that, after adjusting for stage, the prognosis is not influenced by CRC age[13,66], with no difference in stage-specific 5-year disease free survival and OS rates[66].

On the contrary, there also reports from single institution studies that showed poor clinical outcomes for EO-CRC and some authors suggest that the worse survival described for EO-CRC is caused by adverse histopathological features[105,106]. O'Connell *et al*[12]'s review found the average overall 5-year survival for young patients to be 33.4%, consistent with other reports[107,108]. When comparing stage-for-stage survival for younger *vs* older patients with CRC, it seems that young patients with earlier stages have better survival than older patients with the same stage disease. On the contrary, EO-CRC patients with advanced stages do the same or worse than older patients with the same advanced stage. This may justify the described overall lower 5-year survival for EO-CRC because these patients frequently present advanced disease, possibly as a consequence of the delayed diagnosis and/or the more aggressive underlying nature of the disease[12].

Regarding advanced and metastatic disease, studies found that EO-CRC patients have a lower progression-free survival although this does not impact the relative risk of death or OS compared with older CRC patients[109]. As for the mortality rate, although CRC mortality is declining overall and, from 2000 to 2014, CRC death rates decreased by 34% in individuals older than 50 years old, it increased by 13% in EO-CRC patients[110]. One limitation of the studies is that survival outcomes for EO-CRC are often confounded by the inclusion of hereditary colorectal carcinomas, such as LS related CRCs, that are believed to present a better survival compared with non-syndromic colorectal carcinomas[111,112].

SCREENING

CRC incidence and mortality for people older than 50 years old have been declining and the main proposed reason for that is the successful implementation of CRC screening, with a possible contribution of a change in lifestyle. Screening programmes have mostly emerged over the past few decades and generically apply to adults starting from 50 to 60 years old[113]. A recent study shows that from 36

countries from 5 continents, only 4 (Costa Rica, Cyprus, India and Philippines) lack any form of screening programmes[10]. Additionally, only 4 from the 36 mentioned countries have an earlier age cut-off for screening: Italy, beginning at 44 years; China, Japan and Austria, where it begins at 40 years old[113].

On the contrary, prevalence of EO-CRC is increasing. Although one reason for this increase could be over detection through screening, that seems unlikely since screening before age 50 is rare in the general population in most countries[113]. This was the reality until the most recent publication of the American Cancer Society (ACS) guidelines that, for the first time, recommend starting CRC screening at 45 years old for the standard-risk population. The previous CRC screening recommendations have been based on randomized controlled trials and prospective cohort studies. Since there is a lack of data on screening below 50 years old, this recommendation was a result of simulation modelling analyses of screening outcomes, that showed a favourable balance between benefits of screening from 45 years old with its potential life-years gained and the related burdens[114].

Although the absolute risk for EO-CRC is still low, it is expected to increase and the disease burden for younger patients is already substantial and potentially long-term. Additionally, current data show that 40-45 years old adults have the same prevalence of adenomas - CRC precursor lesions[115] - than adults 50-54.5 years old and people aged 40-49 years old account for 75% of the EO-CRC patients[116]. This questions the current age threshold and supports the ACS recommendation.

The debate about lowering the screening age is ongoing, as reviewed in Anderson and Samadder [117]. Those who are favourable to this approach prioritize the need to control the increase in EO-CRC. A study on screening worldwide, showed that two (Austria and Italy) of the only three countries where EO-CRC has declined have screening programs that begin below 50 years old (Italy at 44 and Austria at 40)[113]. Curiously, in Austria, the decreasing incidence of EO-CRC has only occurred in the 40 to 49 years age group[10]. Although another study showed that the same happened in Italy, other described an incidence decrease among the 20-39 years old patients[24].

Reasons for opposing the new 45 years old age threshold are diverse. One argument is that studies have shown that the biggest increase rate in EO-CRC was observed for people aged 20 to 39 years old, an age sector still not included in the suggested screening program. Another argument is that, although increasing, the absolute numbers of EO-CRC are still low, not relevant enough for a screening strategy change. Other concerns are related to the risk of augmenting health inequities related to access to health care services as well as for excessive financial health care costs, especially considering most European countries are still struggling to finance the current screening programs or are still in the process of implementing them[24].

Other authors suggest waiting for adequate randomized screening studies to test the ACS recent recommendation before adopting it and even question if the screening strategy should be applied to a malignancy whose biological and molecular substrate is not fully understood[95], which could mean that standard screening methods and prevention may not even be appropriate for it.

Another small study, based on the evidence that EO-CRC affects predominantly the left side of the colon, suggests a screening strategy with flexible sigmoidoscopy to be applied to people from 40 to 49 years old, as their study group observed that 80% to 83% of those tumours were theoretically within the reach of a flexible sigmoidoscopy[67]. From a different angle, another important factor when reflecting on EO-CRC diagnosis is that only half of the EO-CRC patients with germline pathogenic mutations reported a CRC diagnosis in a FDR and, consequently, were not eligible for high-risk CRC screening [63]. This contributes to the recommendation of performing genetic testing in all EO-CRC. Moreover, tumour testing for MSI or immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 is recommended for all EO-CRC patients, even in countries where universal CRC MSI screening is still not implemented [3].

Another important aspect is the need to alert the medical community for the increasing incidence of EO-CRC and the consequent need for a rigorous assessment of CRC family risk trough detailed and complete family history in medical records, namely reviewing the familiar history of cancer including first- and second-degree relatives as well as personal and familiar history of advanced polyps[82]. Such systematic evaluation may be difficult to reliably execute in real world clinical practice, but it allows an appropriate determination of the patient risk for CRC and the identification of high-risk patients who should be offered earlier screening. Additionally, existing genetic risk models can also be employed and help identifying patients that benefit from genetic testing[118].

Another debatable option is to offer EO-CRC patients next-generation sequencing technology for genetic germline testing. Multigene panel testing has increased detection of germline mutations[79] in a feasible and more cost-effective way than single gene testing[82]. Recently, the National Comprehensive Cancer Network guidelines proposed offering multigene panel testing to all EO-CRC, aiming to increase opportunities for primary and secondary cancer prevention[83].

The disadvantage of this approach is that by testing more genes there is a higher risk of finding genetic variants of unknown significance or without a clear management evidence-based guideline[3]. As so, a multidisciplinary discussion with the active participation of a genetics specialist is recommended to appropriately offer and interpret genetic results, hence providing an appropriate counselling to EO-CRC patients and their families[3].

SPECIFIC CONSIDERATIONS IN THE YOUNG

When dealing with younger patients with a malignancy, there are some extra needs that should be remembered and appropriately managed. EO-CRC patients, as older ones, may need to deal with adverse effects of the different treatment modalities. They face a higher rate of sexual dysfunction than the general population and report to be less sexually active after surgery[119]. Although surgery for CRC does not generally affect fertility[3], chemotherapy, particularly fluorouracil, may reduce sperm count or cause amenorrhea and radiotherapy may also reduce fertility in both men and women[120].

Fertility may play a central concern in the management of EO-CRC patients since they may still be in the reproductive age and have incomplete reproductive plans. As so, patients should be referred to reproductive specialists because options for fertility preservation such as banking of cryopreservation of sperm/oocytes/embryos is recommended prior to gonadotoxic chemotherapy and should be discussed, ahead of starting treatment[121].

Other aspect to consider in EO-CRC patients is the possibility of the diagnosis and/or treatment to be coincidental with a pregnancy, especially with the common delay in childbearing age in the developed world. In that scenario, a multidisciplinary approach, weighting the risks and benefits for both the mother and the foetus should be employed. Although studies in pregnant women are missing, it is consensually agreed that the first trimester is the most vital period to avoid, if possible, systemic agents [122]. Besides fertility, another concern for EO-CRC is to cope with the eventual need for transient or permanent ostomy. The present or past history of an ostomy can contribute to worse sexual function and body image[123].

Additionally, EO-CRC patients, especially the ones submitted to multimodality therapy, will live the rest of their lives under the threat or burden of long-term sequelae. Long-term survivors have reported sustained functional deficits and symptoms, such as sexual disfunction, anxiety, worse body image, embarrassment with bowel movements and faecal incontinence[124], with an impact on their quality of life. Concerning the psychosocial dimension, higher depression and anxiety diagnosis have been described for CRC survivors[125]. Concordantly, the American Society of Clinical Oncology recommends that all cancer survivors should be periodically evaluated for symptoms of depression and anxiety using validated measures and adequately managed, if necessary with the referral for psychological or psychiatric consultation[126]. All of these afore-mentioned factors contribute to the EO-CRC burden of disease, diminish patients' quality of life and should be appropriately addressed throughout patients' life.

CONCLUSION

By 2030, previsions are that more than 1 in 10 colon cancers and 1 in 4 rectal cancers will be diagnosed in patients younger than 50 years old[5]. These numbers are alarming and, consequently, as EO-CRC incidence increases, many studies have tried to shed light on this entity, especially comparing it to the traditional older patient CRC. Evidence suggests that EO-CRC differs from older-onset CRC in its clinical characteristics, pathological features, aggressive behaviour, and molecular profiles. These may be responsible for different responses to treatment and survival, although further studies are needed.

Although a higher proportion of cases, comparing to older patients, is genetic or familial, the majority of EO-CRC cases is still probably related to somatic genetic mutations or epigenetic alterations induced by modifiable risk factors. This is consistent with the birth-cohort effect observed. The contribution of lifestyle risk factors stands as an opportunity for implementing primary prevention measures for CRC, such as the practice of physical activity, having a healthy diet, quitting smoking and limiting alcohol consumption, also preventing obesity and diabetes. A study showed that risk factor modification could diminish CRC-related mortality in about 12% over 20-years[2].

This paper also aims at raising awareness of all the clinicians for the need to lower the suspicion threshold for young patients presenting with alarming gastrointestinal symptoms. We also stress the need to obtain complete clinical information, such as history of polyps, familial history (first- and second-degree relatives) and personal diseases history, namely previous radiation therapy or IBD. Only with this information can the genetic risk of EO-CRC be properly evaluated and subsequent genetic consultation be asked for, when indicated.

EO-CRC patients have some peculiarities and clinicians should be conscious of them. Financial burden and treatment sequelae such as sexual dysfunction, incontinence, anxiety and depression must be considered and managed. Patient's reproductive plans should also be discussed. To conclude, although currently we already have some important data about EO-CRC, larger studies with longer follow-up of these patients are needed in order to reach consensus about the nature and pathogenesis of this disease.

Namely, ongoing studies and clinical trials, by our group and several others across the world, on the impact on survival of increasing clinician awareness on EO-CRC, on the definition of potentially specific phenotypes of EO-CRC, or on their specific responses to radio-, chemo- or immunotherapy will shed light on the subject. Hopefully, by improving young patients' diagnosis, diminishing its observed delay,

tailoring its treatment and follow-up and implementing modifications in lifestyle risk factors, in the future EO-CRC incidence will follow CRC incidence pattern and both entities' burden will decrease.

FOOTNOTES

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Country/Territory of origin: Portugal

ORCID number: Margarida R Saraiva 0000-0002-6750-1112; Isadora Rosa 0000-0002-2953-5987; Isabel Claro 0000-0002-1860-6161.

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MINIREVIEWS

Hamartomatous polyps: Diagnosis, surveillance, and management

Leva Gorji, Peter Albrecht

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Leva Gorji, Peter Albrecht, Department of Surgery, Kettering Health Dayton, Dayton, OH 45405, United States

Corresponding author: Leva Gorji, DO, Doctor, Department of Surgery, Kettering Health Dayton, 405 West Grand Avenue Dayton, Dayton, OH 45405, United States.
leva.gorji@ketteringhealth.org

Abstract

Hereditary polyposis syndrome can be divided into three categories: Adenomatous, serrated, and hamartomatous polyps. Hamartomatous polyps, malformations of normal tissue presenting in a disorganized manner, are characterized by an autosomal dominant inheritance pattern. These syndromes exhibit hamartomatous gastrointestinal polyps in conjunction to extra-intestinal manifestations, which require conscientious and diligent monitoring. Peutz-Jeghers syndrome, Cowden syndrome, and juvenile polyposis syndrome are the most common displays of hamartomatous polyposis syndrome (HPS). Diagnosis can be pursued with molecular testing and endoscopic sampling. Early identification of these autosomal dominant pathologies allows to optimize malignancy surveillance, which helps reduce morbidity and mortality in both the affected patient population as well as at-risk family members. Endoscopic surveillance is an important pillar of prognosis and monitoring, with many patients eventually requiring surgical intervention. In this review, we discuss the diagnosis, surveillance, and management of HPS.

Key Words: Hamartomatous polyps; Peutz-Jegher syndrome; Cowden syndrome; Juvenile polyposis syndrome

Core Tip: We hope that our review article, "Hamartomatous polyps: Diagnosis, surveillance, and management", will function as a concise review of the literature as pertaining to the diagnosis, surveillance, and treatment of the most common hamartomatous polyp syndromes.

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INTRODUCTION

The national cancer institute defines a harmartoma as, "a benign growth made up of an abnormal mixture of cells and tissues normally found in the area of the body where the growth occurs"[1,2]. Diagnosis may be pursued based on personal or family history, endoscopic evaluation, and histopathology identified on endoscopic tissue sampling. The most common disease processes associated with hamartomatous polyps include Peutz–Jeghers syndromes (PJS), Cowden syndrome (CS), and juvenile polyposis syndrome (JPS). While hamartomatous polyps themselves are benign, diligent surveillance is imperative as a significant risk of malignant transformation exists. Additionally, the disease process is often associated with an increased risk of other additional malignancies. As a result, management often requires multidisciplinary evaluation[1,3]. In this review, we discuss the diagnostic considerations, clinical manifestations, gastrointestinal (GI) and extra-intestinal surveillance recommendations, and management options of the most common syndromes associated with hamartomatous polyps.

PJS

Diagnosis

PJS is an autosomal dominant mutation of the mammalian target of rapamycin (mTOR) pathway as a result of a germline mutation of the serine-threonine kinase (*STK11/LKB1*) tumor suppressor gene[4]. The *STK11/LKB1* gene is located on chromosome 19p13.3 and plays an important role in the secondary messenger pathway, which modulates cellular proliferation, responds to cellular energy deficits, and controls cellular polarity[5-7]. The incidence of PJS is estimated to range between approximately 1:8300 and 1:200000 with nearly 70% of cases demonstrating familial inherence and 30% of cases arising from a sporadic mutation[3,8,9]. Although there is often variable penetrance and clinical heterogeneity, diagnostic clinical criteria include: Any number of polyps with a family history of PJS, two or more Peutz-Jeghers polyps (PJPs), mucocutaneous pigmentations with family history of PJS, or any number of PJPs with mucocutaneous pigmentation[4,8,10]. While endoscopically similar to other polyps, PJPs have a characteristic phyllodes appearing epithelial component and a cystic glandular component which extends into the submucosa and muscular propria on hematoxylin and eosin staining[7,11-13]. Notably, PJPs are exclusively located within the small bowel. Gastric polyps associated with PJS have similar characteristics to hyperplastic gastric polyps and are not considered PJPs[7,12]. The median age of initial polyp development is 12 years-old, with nearly half of patients experiencing symptoms by the age of 20 [7,14]. The primary clinical manifestation of PJS is chronic bleeding from GI polyps leading to anemia. Small bowel obstruction secondary to an intussusception from a hamartomatous polyp of the GI tract occurs in up to 70% of patients, with the average age of diagnosis being 23 years-old[7]. Patients also characteristically present with mucocutaneous hyperpigmentation surrounding the oral cavity, eyes, nostrils, or surrounding the anus[15].

Surveillance

Due the increased risk of GI and extra-intestinal malignancies, a multidisciplinary approach to screening is encouraged. While the mean age of index cancer diagnosis is 41 years old, PJS is associated with an overall cumulated malignancy rate of 90% by the age of 70[4,8]. GI malignancies commonly associated with PJS include gastric, intestinal, and the pancreatic. Additional extra-intestinal malignancy manifestations include breast, endometrial, ovarian, and lung[11]. Multiple studies have been pursued in order to further characterize the malignancy risk that is associated with PJS (Table 1). The National Comprehensive Cancer Network, European Hereditary Tumour Group, and the American College of Gastroenterology (ACG) provide surveillance recommendations for patients[16-19]. A meticulous regimen of lifelong malignancy surveillance is imperative (Table 2).

Management

Endoscopy is an essential modality of surveillance and management of polyps associated with PJS. Polypectomy during esophagogastroduodenoscopy (EGD) and colonoscopy is particularly appropriate for patients with a limited number of polyps ranging from 0.5-1 cm in size. If the polyposis that is encountered is too large or numerous to be managed endoscopically, surgical resection of the diseased segment may be pursued[18,20].

Table 1 Studies investigating the malignancy risk associated with Peutz-Jeghers syndrome

Ref.	*N*	Location	Outcome
Chen *et al*[80]	336	China	Patients with PJS possess a 50% cumulative malignancy risk by the age of 60, with the most common malignancy being colorectal cancer at a median age of 41
Hearle *et al* [81]	419	United Kingdom	The risk of developing a malignancy is 85% at the age of 70 in patients with PJS, most common being gastrointestinal in origin
Ishida *et al* [82]	583	Japan	Patients with PJS possess a cumulative malignancy risk of 83% by the age of 70, with an increased rate of gynecologic malignancy in comparison to previously reported data
Korsse *et al* [83]	144	Netherlands	The cumulative risk of pancreatico-biliary malignancy is 32% by the age of 70 in patients with PJS
Mehenni *et al* [84]	149	Switzerland	Patients with PJS have a cumulate malignancy risk of 67% at age 70, particularly with *STK11/LKB1* mutations in exon 6. Malignancies most commonly occur in the GI tract
Resta *et al*[11]	119	Italy	The *STK11/LKB1* mutation is associated with a relative overall cancer risk of 15.1, with pancreatic and cervical malignancies being the most common; median age of diagnosis noted to be 41 yr
Van Lier *et al* [8]	133	Netherlands	Patients with PJS possess a cumulative malignancy rate of 76% by the age of 70; malignancies most commonly occur in the GI tract and in women

GI: Gastrointestinal; PJS: Peutz-Jeghers syndrome.

Table 2 Malignancy screening for Peutz-Jeghers syndrome[4,8,10,16-18,85,86]

Malignancy	Screening
Breast	Annual self-breast exam beginning at the age of 18. Annual breast exam with MRI or mammography beginning at the age of 25, and as needed
Cervical	Cervical smear annual beginning at the age of 20, and as needed
Colon	Colonoscopy every 1-3 yr beginning at the age of 8, and as needed. Routine surveillance at 18 if baseline endoscopy at age 8 is negative
Pancreas	Annual endoscopic ultrasound or MRCP/ERCP beginning at the age of 30, and as needed
Stomach	EGD every 1-3 yr beginning at the age of 8, and as needed. Routine surveillance at 18 if baseline endoscopy at age 8 is negative
Small bowel	Capsule endoscopy or CT/MRI enterography every 2-3 yr, beginning at the age of 8, and as needed
Testicular	Annual testicular exam and ultrasound beginning at birth
Uterus	Annual pelvic ultrasound and exam beginning at the age of 25, and as needed; consider total hysterectomy once complete with child-bearing

MRI: Magnetic resonance imaging; CT: Computed tomography; MRCP: Magnetic resonance pancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastroduodenoscopy.

Management of small bowel polyps includes double balloon enteroscopy (DBE) or surgery. DBE allows for diagnosis and treatment of polyps located in the jejunum and the ileum. The device possesses a 200 cm long enteroscope with a 145 cm long overtube. There are two latex balloons attached to the tip of the endoscope and overtube, which are managed with a pressure-control pump system serving to prevent redundancy of the small bowel[21-23]. With this approach, manipulation and advancement of the scope may be occasionally impaired in patients who have previously undergone abdominal surgery secondary to intraabdominal adhesions, warranting intraoperative enteroscopy[24,25]. The European Society of Gastrointestinal Endoscopy (ESGE) recommends elective polypectomy for small bowel polyps that are > 15-20 mm in size to reduce the risk of intussusception. In symptomatic patients, polyps that are < 15 mm should be removed, as well[19]. Complications from DBE occur in less than < 1% of cases, but include perforation, post-procedure hemorrhage, pancreatitis and aspiration[23,26]. DBE is an effective method for early detection and non-operative removal of polyps located in the small bowel[18,27]. Similar to polyposis of the colon, surgical resection may be required in the setting of numerous or large polyposis of the small bowel[19].

Surgical intervention is required for patients with PJS who present with intussusception, a phenomenon where the proximal segment of bowel telescopes into a distal segment of bowel with the polyp acting as a lead point. If no intervention is pursued, bowel ischemia and perforation may occur [4]. Patients with PJS possess a cumulative risk of intussusception reaching nearly 44% by the age of 10,

which increases to nearly 50% by the age of 20[20]. The risk of intussusception is greatest with polyps that are 15 mm or larger[28]. Intraoperatively, frankly ischemic bowel evidently must be removed; however, polypectomy without bowel resection is recommended in cases of reversible ischemia. As the risk of recurrent intussusception is present especially in younger patients, some authors have recommended further evaluate the bowel through manual palpation or enteroscopy *via* an intraoperative enterotomy in order to remove any additional polyps greater than 15 mm[20,29].

According to the ESGE, pancreatic lesions should be addressed with partial pancreatectomies in the case of: (1) Solid lesions ³10 mm unless known to be benign; or (2) intraductal papillary mucinous neoplasms with high-risk features including mural nodules, enhanced solid component, obstructive symptoms, enhanced cyst wall, abrupt changes to duct size, and main pancreatic duct ³10 mm[19,30].

Breast lesions should be managed based on current breast cancer guidelines, as no therapeutic strategy specific to PJS is presently available. A mastectomy for the purpose of risk reduction should be discussed in a multi-disciplinary setting accounting for additional patient risk factors and family history [19]. Similarly, while an established malignancy risk exists between PJS and cervical, uterine, and testicular cancer, no formal intervention guidelines specific to the correlation exists.

Studies have also demonstrated that the immunosuppressant agent Sirolimus (Rapamycin) has led to reduction in the number and size of polyps in a mouse model, which is largely attribute to the anti-angiogenic properties of the drug[31-33]. Currently, phase 4 prospective study for characterization of the drug in patients with PJS is being undertaken.

CS

Diagnosis

CS is an autosomal dominant mutation in the *phosphatase and tensin homolog* (*PTEN*) gene impacting the downregulation of the mTOR pathway. Consequently, the *PTEN* regulation of cellular proliferation and survival is impaired[34,35]. The incidence of CS is estimated to be roughly 1:200000[36]. CS characteristically presents with hamartomatous polyps which may appear in any organ. Nearly all patients exhibit some type of skin lesion including trichilemmomas, acral keratoses, and papillomatous papules. Additionally, nearly 80% of patients demonstrate GI hamartomatous polyps and 80% of patients exhibit macrocephaly. Diagnosis is based on the guidelines established by the International CS Consortium: Patients must possess two major criteria one of which must be either macrocephaly or Lhermitte-Duclos disease (LDD), one major criteria with three minor criteria, or four minor criteria (Table 3)[37]. 40% of CS is associated with LDD-a rare, slow growing dysplastic gangliocytoma of the cerebellum manifesting with a broad range of clinical findings. The presentation of LDD may range from being asymptomatic to exhibiting signs of ataxia, cranial nerve palsy, vertigo, mental impairment and deterioration, intracranial hypertension, and hydrocephalus. The wide spectrum of clinical manifestations is attributable to the slow growing nature of the neoplasm[38,39].

Surveillance

The majority of patients with CS are at risk for developing breast, thyroid, renal, endometrial, colorectal, and skin malignancies (Table 4). Given this established predisposition, attentive multidisciplinary surveillance and screening is an essential component of disease management (Table 5). The purpose of these surveillance recommendations is early detection in order to allow curative oncologic treatment.

Management

There is no available curative treatment for CS. Rather, management is geared towards early detection of malignancy and timely oncologic treatment. Genetic counseling is also prudent in order to promote early detection and surveillance for kindred. Options for treatment of cutaneous lesions include, surgical excision, 5-Flurouracil, carbon dioxide laser treatments, isotretinoin[40,41]. Polyps found on endoscopy may be removed endoscopically, if amenable. Otherwise, identified malignancies should be addressed utilizing the most up-to-date therapeutic regimens.

Associated syndromes

PTEN hamartoma tumor syndrome (PHTS) is a broad category of hamartomatous pathologies arising as pathogenic *PTEN* mutation variants identified by gene-targeted testing and comprehensive genomic testing. PHTS classically includes CS, Bannayan-Riley-Ruvalcaba Syndromes (BRRS), PTEN-related Proteus Syndromes (PS), and *PTEN*-related Proteus-like syndromes (PLS). Notably, however, segmental overgrowth lipomatosis arteriovenous malformation epidermal nevus (SOLAMEN) syndrome also shares the same mutation[42,43]. It has been proposed that CS and BRRS may simply be varying spectrums of the same syndrome[44]. While formal diagnosis criteria are not present for BRRS, patients often present with macrocephaly, penile mucocutaneous lesions, delayed psychomotor development, and visceral hamartomas[44,45]. Approximately half of patients with BRRS have GI polyposis, with an analogous histopathologic presentation[44]. Nearly 60% of patients with BRRS possess a germline *PTEN*

Table 3 Major and minor criteria for the diagnosis of Cowden syndrome[37]

Major criteria	Minor criteria
Breast malignancy	Fibrocystic changes of the breast
Lhermitte-Duclos disease	Fibromas
Macrocephaly	Gastrointestinal hamartomas
Thyroid malignancy	Genitourinary tumors or malformations
	Lipomas
	Mental retardation
	Thyroid lesions, *i.e.* goiter

Table 4 Studies investigating the malignancy risk associated with *phosphatase and tensin homolog* mutation

Ref.	*N*	Location	Outcome
Tan *et al*[87]	3399	North America, Europe, and Asia	Patient with *PTEN* mutations possess a lifetime breast cancer risk of 85%, thyroid cancer risk of 35%, renal cell carcinoma of 35%, endometrial cancer risk of 28%, colorectal cancer risk of 9%, and melanoma risk of 6%
Fackenthal *et al*[88]	2	United States	There is an increased risk of male breast cancer in patient with *PTEN* mutations and CS
Harach *et al* [89]	11	Argentina	Patients with CS have increased likelihood of developing benign thyroid lesions, with increased risk of malignant transformation
Ngeow *et al* [90]	2723	United States	CS and CS-like phenotypes possess standardized incidence rate for thyroid cancer of 72%, particularly follicular thyroid cancer
Heald *et al*[91]	127	North America and Europe	*PTEN* mutations are associated with early-onset (age < 50) colorectal malignancy; routine colonoscopy should be encouraged

PTEN: *Phosphatase and tensin homolog*; CS: Cowden syndrome.

Table 5 Malignancy screening for *phosphatase and tensin homolog* mutation[92]

Malignancy	Screening
Breast	Annual self-breast exam at the age of 18; annual clinical breast exam at the age of 25; annual MRI at the age of 30
Colon	Colonoscopy every 5 yr at the age of 35, and as needed
Endometrial	Annual exam with biopsy at the age of 35, and as needed. Hysterectomy upon childbearing completion
Kidney	Annual renal ultrasound at the age of 40; CT or MRI as indicated
Skin lesion	Annual dermatologic exams, no consensus on initiation age
Thyroid	Annual thyroid ultrasound at the age of 7

MRI: Magnetic resonance imaging; CT: Computed tomography.

mutation[46]. SOLAMEN syndrome is also associated with hamartomatous polyps, macrocephaly, lipomas, and hemangiomas. These patients further possess an increased risk of skin, GI, thyroid, breast, brain, and genito-urinary malignant neoplasms[47,48]. Surveillance is advised to be similar to CS, given the mutual *PTEN* mutation association (Table 5). Treatment is predominantly symptomatic and often requires a multidisciplinary approach[49].

PS and PLS are similar pathologic processes that may be associated with a *PTEN* mutations[50]. PS has been associated with *AKT1* and *PTEN* mutations, both of which influence the PI3KCA/AKT pathway[51]. Pulmonary embolism secondary to a hypercoagulable state and pneumonia are noted to be contributing factors to early mortality in this patient population. Other complications include scoliosis, central nervous system abnormalities, ophthalmic complications, abnormal bone growth, and pulmonary malformations[52]. Treatment is often targeted toward the correction of the functional limitations caused by the skeletal deformities associated with the disease. Patients should be closely monitored for the development of venous thrombosis; however, no recommendations for prophylactic anticoagulation currently exist[3,53]. Patients that have presentations similar to PS, but do not possess

all the diagnostic criteria are deemed to have PLS.

JPS

Diagnosis

JPS is an autosomal dominant condition resulting from a germline mutation of the *SMAD4* or *BMPR1A* genes in 60% of the affected population. These genes impact the transforming growth factor-beta signaling pathway[54,55]. The estimated incidence of JPS is approximately between 1:100000 to 1:160000 [56,57]. Clinical diagnosis is based on the presence of one of the following: 5+ juvenile polyps in the colorectal region, 2+ juvenile polyps in the GI tract, or any number of juvenile polyps with a family history of juvenile polyposis[18,56,58,59]. Juvenile polyps refer to the histopathology of the polyps, rather than the age of onset. Juvenile polyps are hamartomas demonstrating a dense stroma with a smooth surface and mucin-filled cystic lamina propria[60]. Macroscopically, these pedunculated polyps have an erosive surface ranging from 5 mm to 50 mm in size. While distinction with sporadic juvenile polyps is difficult to make, JPS polyps frequently demonstrate epithelial neoplastic transformation, reduced stroma, reduced dilated glands, and increased proliferative glands[61].

Subtypes of JPS include JPS of infancy, generalized JPS, and juvenile polyposis coli. JPS of infancy is characterized by sessile or pedunculated polyps diffusely throughout the GI tract ranging from 1-30 mm. Although further investigation must be pursued, some sources have reported JPS of infancy is caused by mutations resulting in the continuous deletion of *BMPR1A* and *PTEN*[62]. The disease process is plagued with early mortality secondary to hemorrhage, malnutrition caused by diarrhea, and intussusception[61,63]. Considered to be a variable spectrum of penetrance of the same disease, generalized JPS occurs diffusely throughout the GI tract while juvenile polyposis coli is limited to the colorectal region[64]. Nearly half of the patients with generalized JPS or juvenile polyposis coli possess a heterozygous germline mutation of the *SMAD4* or *BMPR1A* gene[64].

Extra-intestinal manifestations are present in up to 15% of patients including midgut rotation, cardiac abnormalities, and craniofacial abnormalities. JPS may also be present in conjunction to hereditary hemorrhagic telangiectasia (HHT) syndrome[65,66]. Patients with HHT have a characteristic increased risk of bleeding secondary to greater rates of arteriovenous malformations of the lung, liver, brain, and GI tract. HHS is an autosomal dominant disorder also associated with a *SMAD4* gene mutation[65,67,68]. Patient may demonstrate varying degrees of disease qualities when they have both disorders simultaneously. However, the combination of both disorders results in a higher risk of colorectal malignant neoplasms and anemia[68,69]. Although further studies are required, the current perspective on the malignant pathogenesis of JPS includes the "landscaper mechanism" and the "gatekeeper" model. The "landscaper mechanism" postulates that genetic alterations lead to an abnormal stromal environment, which leads to malignant transformation of neighboring epithelium. A de novo study by Haramis *et al*[70] demonstrated that bone morphogenetic protein (*BMP*)-4 inhibition, a common JPS pathway mutation, results in ectopic crypt units adjacent to the crypt-villus axis supporting the "landscaper mechanism"[70]. A "gatekeeper" model has also been proposed, where the biallelic *SMAD4* mutation contributes to malignant transformation and progression[71,72].

Surveillance

JPS has an established increased risk of GI malignancy, with a cumulative lifetime risk reported to range between 39%-63%[73,74]. The *SMAD4* gene has been associated with a greater risk of GI malignancies as well as a greater number of polyps in comparison to the *BMPR1A* mutation[54,75,76]. JPS is associated with an elevated risk of colorectal and gastric malignancies (Table 6). Therefore, a combination of colonoscopy and EGD is recommended for appropriate screening and surveillance[77] (Table 7).

Management

Endoscopic management is typically feasible for patients with a limited number of polyps[18]. EGD may be utilized for surveillance and endoscopic removal of polyps located in the stomach and duodenum. In the setting of extensive polyposis, advanced dysplasia, or malignancy, a partial or complete gastrectomy may be pursued[44,78] Jaoude *et al*[55] recommended proposed prophylactic gastrectomy in patients with symptomatic JPS, asymptomatic patients with a *SMAD4* mutation, or patients with unmanageable polyposis defined as > 50-100 polyps[55]. Certainly, morbidity and mortality are greater with surgical intervention. However, surgical intervention must be weighed against the possibility of inadequate surveillance and treatment with endoscopy, particularly in the setting of an elevated likelihood for sporadic gastric malignancy associated with the *SMAD4* gene mutation. Further investigation should be pursued to determine the optimal parameters for surgical intervention, particularly in the prophylactic setting.

Small bowel polyps should be evaluated periodically with enteroscopy. Patient with limited small bowel polyps may be managed with endoscopic polypectomy but will require surgical treatment in the event of obstructive symptoms associated with intussusception. Colonoscopy is an essential screening and management tool for colonic polyps. However, in the setting of numerous polyps, advanced

Table 6 Studies investing the malignancy risk associated with juvenile polyposis syndrome

Ref.	N	Location	Outcome
Brosens *et al*[73]	84	United States	Patients with JPS possess a lifetime cumulative risk of 38.7% for development of colorectal cancer, with the average age of diagnosis being 43.9
Latchford *et al*[56]	44	United Kingdom	The *SMAD4* gene is a poor prognostic indicator for development of gastric malignancy. Colonic polyps have predominance to ascending colon
Howe *et al*[93]	117	United States	Kindred of patients with JPS possess approximately 50% cumulative risk of developing gastric malignancy

JPS: Juvenile polyposis syndrome.

Table 7 Malignancy screening for juvenile polyposis syndrome[18,77,94-96]

Malignancy	Screening
Colorectal	Colonoscopy every 1-3 yr between the ages of 12-15; screening should be initiated sooner if earlier onset of symptoms
Gastric	Esophagogastroduodenoscopy every 1-3 yr between the age of 12-15; screening should be initiated sooner if earlier onset of symptoms
HHT mutation	Brain MRI, cardiac echocardiogram, testing for lung arterio-venous malformations

HHT: Hereditary hemorrhagic telangiectasia; MRI: Magnetic resonance imaging.

dysplasia, or malignancy, a colectomy may be pursued. Surgical options include proctocolectomy with ileal pouch anal anastomosis (IPAA) or colectomy with ileorectal anastomosis (IRA) based on rectal involvement. Nearly half of patients who elect to undergo IRA will require additional proctocolectomy with IPAA secondary to the development of rectal polyposis[18,44,78].

An area of potential emerging investigation includes the utility of chemoprevention in patients with JPS. Van Hattem *et al*[79] determined an increased expression of cyclooxygenase-2, particularly in patients with a *BMPR1A* gene mutation. Further investigation should be pursued in order to determine the clinical implications of this expression profile[79]. Ultimately, additional studies should be pursued regarding the optimal management of these patients.

CONCLUSION

Hamartomas polyps result from hyperproliferations of normal tissues and are often associated with genetic mutations. While patients experience symptoms from the hamartomas themselves, there also exists an established malignancy risk associated with these disorders. Therefore, patient education regarding surveillance is imperative, and a multidisciplinary approach is often required for comprehensive management. First degree relatives should also be investigated for the diagnosis due to the dominant inheritance patterns that these disease processes possess. Due to the rarity of the disorders, limited studies are available on the clinical and molecular aspects of the disease processes, and further investigation should be dedicated to understanding the pathology associated with these syndromes.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Leva Gorji 0000-0001-6837-5317.

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ORIGINAL ARTICLE

Basic Study

Stress granules inhibit endoplasmic reticulum stress-mediated apoptosis during hypoxia-induced injury in acute liver failure

Wen-Yuan Li, Fan Yang, Xun Li, Lu-Wen Wang, Yao Wang

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Wen-Yuan Li, Department of Anesthesiology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Fan Yang, Xun Li, Lu-Wen Wang, Yao Wang, Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Corresponding author: Yao Wang, Doctor, Chief Physician, Department of Infectious Diseases, Renmin Hospital of Wuhan University, No. 238 Jiefang Road, Wuhan 430060, Hubei Province, China. rm003743@whu.edu.cn

Abstract

BACKGROUND

Stress granules (SGs) could be formed under different stimulation to inhibit cell injury.

AIM

To investigate whether SGs could protect hepatocytes from hypoxia-induced damage during acute liver failure (ALF) by reducing endoplasmic reticulum stress (ERS) mediated apoptosis.

METHODS

The agonist of SGs, arsenite (Ars) was used to intervene hypoxia-induced hepatocyte injury cellular model and ALF mice models. Further, the siRNA of activating transcription factor 4 (ATF4) and SGs inhibitor anisomycin was then used to intervene in cell models.

RESULTS

With the increase of hypoxia time from 4 h to 12 h, the levels of HIF-1α, ERS and apoptosis gradually increased, and the expression of SGs marker G3BP1 and TIA-1 was increased and then decreased. Compared with the hypoxia cell model group and ALF mice model, the levels of HIF-1α, apoptosis and ERS were increased in the Ars intervention group. After siRNA-ATF4 intervention, the level of SGs in cells increased, and the levels of HIF-1α, ERS and apoptosis decreased. Compared with the siRNA-ATF4 group, the levels of G3BP1 in the siRNA-ATF4+anisomycin group were decreased, and the levels of HIF-1α, ERS and apoptosis were increased. Moreover, compared with the ALF group, the degree of liver injury and liver function, the levels of HIF-1α, ERS and apoptosis in the Ars intervention group were decreased, the level of SGs was increased.

CONCLUSION
SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS-mediated apoptosis.

Key Words: Acute liver failure; Stress granules; Hypoxia; Endoplasmic reticulum stress; Apoptosis

Core Tip: Hepatocytes were damaged by hypoxia and ischemia injury in the process of acute liver failure. At this time, the content of HIF-1α in cells increased, which inhibited the formation of stress granules (SGs) mediated by G3BP1 and promoted the expression of endoplasmic reticulum stress (ERS) marker molecules activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein-homologous protein. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway.

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INTRODUCTION

Acute liver failure (ALF) is a common clinical syndrome in intensive care units, with a high mortality due to various causes, such as drugs, toxic exposure, viral hepatitis, *etc*[1]. ALF can be preliminarily diagnosed in patients with no history of basic liver disease, with rapid deepening of jaundice in a short period of time. It has the typical symptom, such as poor appetite, vomiting, diarrhea, abdominal distension and hepatic encephalopathy. Advanced high bilirubin or aminotransferase, and prothrombin activity of coagulation item ≤ 40% can be the basic diagnosis[2]. For such patients, it is extremely important to be transferred to intensive care unit as soon as possible for mechanical ventilation to improve oxygenation. The comprehensive medical treatment, blood purification (artificial liver) and maintenance of homeostasis in human body should be carried out throughout the treatment. For patients with ALF, liver transplantation is the only completely effective treatment option. However, the development of liver transplantation is limited by shortage of liver source, strict transplantation conditions, huge treatment cost and rejection after transplantation[3].

Endoplasmic reticulum stress (ERS) plays an important role in the regulation of inflammatory response and apoptosis, and severe ERS promotes the occurrence and development of ALF[4]. When cells undergo ERS, they can be induced by type-1 ER transmembrane protein kinase (IRE1), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6), induce unfolded protein response (UPR)[5]. Unfolded proteins can alter cellular transcription and translation programs to regulate protein synthesis, secretion, and degradation to relieve endoplasmic reticulum stress, thereby protecting cells[6]. When ERS exceeds the controllable range of the body, cells will undergo apoptosis. CCAAT/enhancer-binding protein-homologous protein (CHOP) is a transcription factor that controls gene-encoded components in apoptosis[7]. The activation of ERS of the three pathways will eventually induce the expression of the apoptotic factor CHOP[7]. Among them, PERK can activate eIF2a and further induce the expression of ATF4. ATF4 can promote the activation of CHOP, and the highly expressed CHOP triggers the bax/bad system to activate caspase9 and caspase3, which in turn leads to apoptosis[8].

Stress granules (SGs) refer to the fact that when eukaryotic cells are subjected to various environmental stimuli, such as oxidative stress, heat shock, ultraviolet radiation, and viral infection. Aids in the transport of dense granular material formed into the cytoplasm, which is an adaptive regulation mechanism of cells[9]. SGs are a protective mechanism of cells, which is the product of the host's response to external stress. The production mechanism of SGs is related to neurological diseases[10], viral infections[11], and cancers[12], *etc*. There is no study on the relationship between SGs, ERS and hepatocyte apoptosis in ALF. This experiment investigates the effect and mechanism of SGs on the apoptosis process induced by ERS during the hypoxic injury of hepatocytes in ALF.

MATERIALS AND METHODS

Chemicals and reagents

The normal human liver L02 cell line was purchased from the China Center for Type Culture Collection (Wuhan University). DMEM medium and fetal bovine serum (FBS) were purchased from GIBCO (NY, United States). Lipopolysaccharide (LPS), D-galactosamine (D-Gal), arsenite (Ars) and anisomycin were purchased from Sigma (St. Louis, United States). Rabbit anti-human/ mouse G3BP1, T-cell restricted intracellular antigen-1 (TIA-1), ATF4, CHOP, BAX, BCL2, GAPDH antibodies, Cy3-labeled goat anti-rabbit secondary antibody were purchased from Proteintech (Wuhan, China). Rabbit anti-human/ mouse cleaved caspase3 (c-cas3), c-cas9 antibodies were purchased from Cell Signaling Technology (Boston, United States). Goat anti-rabbit fluorescent secondary antibody IRDye800 was purchased from LI-COR Biosciences (Lincoln, United States). Human and mouse lactate dehydrogenase (LDH) and HIF-1α enzyme-linked immunosorbent assay kits were purchased from Elabscience (Wuhan, China). RNAiso Plus, PrimeScript™, RT reagent and SYBR Premix Ex Taq kit were purchased from TaKaRa (Dalian, China). The Annexin V-phycoerythrin/7-aminoactinomycin D (Annexin V-PE/7AAD) apoptosis kit Apoptosis kit was purchased from BD (San Diego, United States). The transfection reagent Lipo200 was purchased from Invitrogen (CA, United States).

Cell culture and treatment

After receiving the L02 cells, they were centrifuged to remove the cell cryopreservation solution. The cells were then added DMEM complete medium (containing 10% FBS), and cultured in a 37 °C 5% CO_2 constant temperature incubator in a suitable humidity environment. The adherence and growth of the cells were observed under a microscope, and the cells were in the logarithmic growth phase for experiments. The hypoxia-treated cell group was placed in a three-gas incubator at 37 °C with a volume fraction of 95% N_2 + a volume fraction of 5% CO_2 for hypoxia treatment for 4 h, 8 h, and 12 h, respectively. Hepatocytes treated with hypoxia for 12 h were used as model group. The Hypoxia + Ars group was first treated with Hypoxia for 12 h, followed by hypoxia treatment for 12 h. Hypoxia + anisomycin + siRNA-ATF4 group cells were first transfected with siRNA, then intervened with anisomycin for 12 h, and finally treated with hypoxia for 12 h. The sequence of siRNA-ATF4 was 5'-TCC CTC AGT GCA TAA AGG A-3'. And the sequence of non-targeting control siRNA was 5'-UUC UCC GAA CGU GUC ACG A-3'. The siRNA was transfected into cells using Lipo2000 according to the manufacturer's instructions.

Animal groups and treatment

All animal experimental procedures were evaluated and approved by the Laboratory Animal Care and Committee of Renmin Hospital of Wuhan University (WDRM20181018) and followed institutional guidelines as well as ARRIVE guidelines. Thirty male C57BL/6 rats were randomly divided into 3 groups, namely the normal group, the model group, and the Ars group, with 10 rats in each group. All mice were acclimated to feeding for 1 wk before the experiment. Referring to the previous modeling method[13,14], the mice in the model group and Ars group were injected with D-Gal 400 mg/kg and LPS 100 μg/kg intraperitoneally. The mice in Ars group were given Ars (1 mg/kg) intraperitoneal treatment 3 d before modeling, once a day, until 24 h after modeling. The rats in the normal group and the model group were given normal saline by gavage every day. 12 h after modeling, the survival of mice in each group was observed and recorded. Body weights and health of the animals were monitored every other day. If the mice were unable to eat or drink, showed any abnormal behavior, or signs of toxicity, pain, or distress, they would be removed from the study. At the end of the experiment, the mice were euthanized by sodium pentobarbital, and death was confirmed by the absence of a heartbeat. About 1 mL of ocular venous blood was collected. Liver tissue was taken, washed with normal saline, and then stored in -80 °C freezer for tissue homogenate, protein and mRNA extraction. The rest of the liver tissue specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and used for hematoxylin-eosin (HE) and transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining.

Cell apoptosis, HIF-1α and LDH detection

Referring to the previous method[15], the apoptosis level of L02 cells in each group was detected by flow cytometry using Annexin V-PE/7AAD apoptosis kit. After intervention in each group, 1.0×10^5 cells were added to 400 μL buffer solution for staining. 5 μL of Annexin V-PE and 5 μL of 7AAD were then added to the cell suspension. The cell was incubated in the dark for 15 min at 37 °C. The apoptosis rate of early and late cells was detected by flow cytometry (BD, United States). According to the description in the previous experiments[16], the cell supernatant, homogenates of cells and liver tissues in each group were taken. Then the HIF-1α and LDH detection kits were used to evaluate the content of HIF-1α in cell or tissue homogenates, LDH in cell supernatant or serum according to the kit instructions.

Determination of G3BP1 expression by immunofluorescence

The cells in each group were modeled and intervened by the above method. After 24 h of incubation, they were fixed with cell fixative for 25 min, and then washed with PBS for 3 times. 0.2% Triton was permeabilized for 20 min, and 0.5% BSA was blocked for 30 min. G3BP1 primary antibody (1:1000) was incubated overnight. The next day, the secondary antibody was incubated for 1 h, nuclei were stained with DAPI for 3 min, washed with PBS for 5 times. The anti-fluorescence quencher was added dropwise, and the slides were mounted and observed under an upright microscope.

Detection of ATF4 mRNA by quantitative real-time polymerase chain reaction

The total RNA of each group of cells was extracted by RNAiso Plus kit. The cDNA was further amplified using Prime-Script RT reagent kit. The amplified cDNA was subjected to quantitative polymerase chain reaction (qPCR) by real-time PCR (RT-PCR) using SYBR Premix Ex Taq kit. The PCR program was set as: initial 95 °C for 10 s; 95 °C for 5 s and 60 °C for 20 s, 40 cycles. ATF4 primer sequences: forward 5'-TCAAACCTCATGGGTTCTCC-3' and reverse 5'-GTGTCATCCAACGT-GGTCAG-3'. GAPDH primer sequences: forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The relative gene expression of ATF4 was calculated by $2^{-\Delta\Delta CT}$. The above methods refer to previous reports[17].

Protein expression was detected by western blot

Referring to the previous experimental method[18], the cells or liver tissues of each group were collected into the centrifuge tube, and were broken under ice bath by ultrasound. After centrifugation at 4 °C, the supernatant was taken. Total protein was determined by bicinchoninic acid method and protein concentration was adjusted. It was then added protein loading buffer, mixed well, and boiled for 10 min. Electrophoresis was performed with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. 10 μL sample to each well was then add. Electrophoresis was performed at 80 V and then at a constant current of 200 mA. PVDF membranes were sealed with 5% skim milk powder at room temperature for 1h. Then primary antibody (G3BP1, 1:1,000; TIA-1, 1:1000; ATF4, 1:1000; CHOP, 1:1000; BAX, 1:1000; BCL2, 1:1000; c-cas3, 1:1,000; c-cas9, 1:1000; GAPDH, 1:3000) was used to incubated the membranes overnight at 4 °C. After the membranes were washed, fluorescent secondary antibody (1:10000) was used to incubate at room temperature without light for 1 h. Finally, the membranes were scanned with Odyssey system to detect and compare the relative protein expression levels of each group.

Biochemical tests and histological studies

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) in serum in each group were detected by automatic biochemical analyzer. Liver tissues fixed by formaldehyde and embedded in paraffin were taken from each group. The paraffin blocks were sectioned. Hematoxylin and eosin were used to stain the slices. The slices were dehydrated and dried in turn. The histopathological changes of liver in each group were observed under inverted microscope. The slices were stained with TUNEL and incubated away from light. After washing with PBS, DAPI was dropped and incubated away from light. Cell morphology was observed under fluorescence microscope, and apoptosis index in each tissue was calculated according to the method described previously[19].

Statistical analysis

SPSS 19.0 statistical software was used for statistical analysis. All data were expressed as mean ± SD. *t*-test was used for comparison between two groups, and analysis of variance test was used for comparison between three groups and more than three groups. $P < 0.05$ means the difference was statistically significant.

RESULTS

Changes of SGs, ERS and apoptosis levels with the change of hypoxia time

The histopathological examination of liver failure revealed massive necrosis and extensive intrahepatic inflammatory reaction, resulting in the embolization of micro vessels and the structural destruction of hepatic sinuses. The microcirculation disturbance could lead to obvious hepatic perfusion obstruction, reflecting ischemic hypoxic injury[20]. The ischemia-hypoxia and ischemia-reperfusion (IR) could further activate local inflammatory response or aggravate local perfusion disorders through platelet activation pathway[21].

In this part, the model of hepatocyte hypoxia *in vitro* was used to simulate the ischemic hypoxia injury during ALF. It was firstly observed how the levels of SGs, ERS and apoptosis changed over time. As shown in Figure 1A-D, compared with the normal group, the apoptosis rate, HIF-1α and LDH contents in the hypoxia group for 4 h were increased ($P < 0.05$). Compared with the 4 h group, the apoptosis rate and the contents of HIF-1α and LDH in hypoxia group for 8 h and 12 h was furtherly

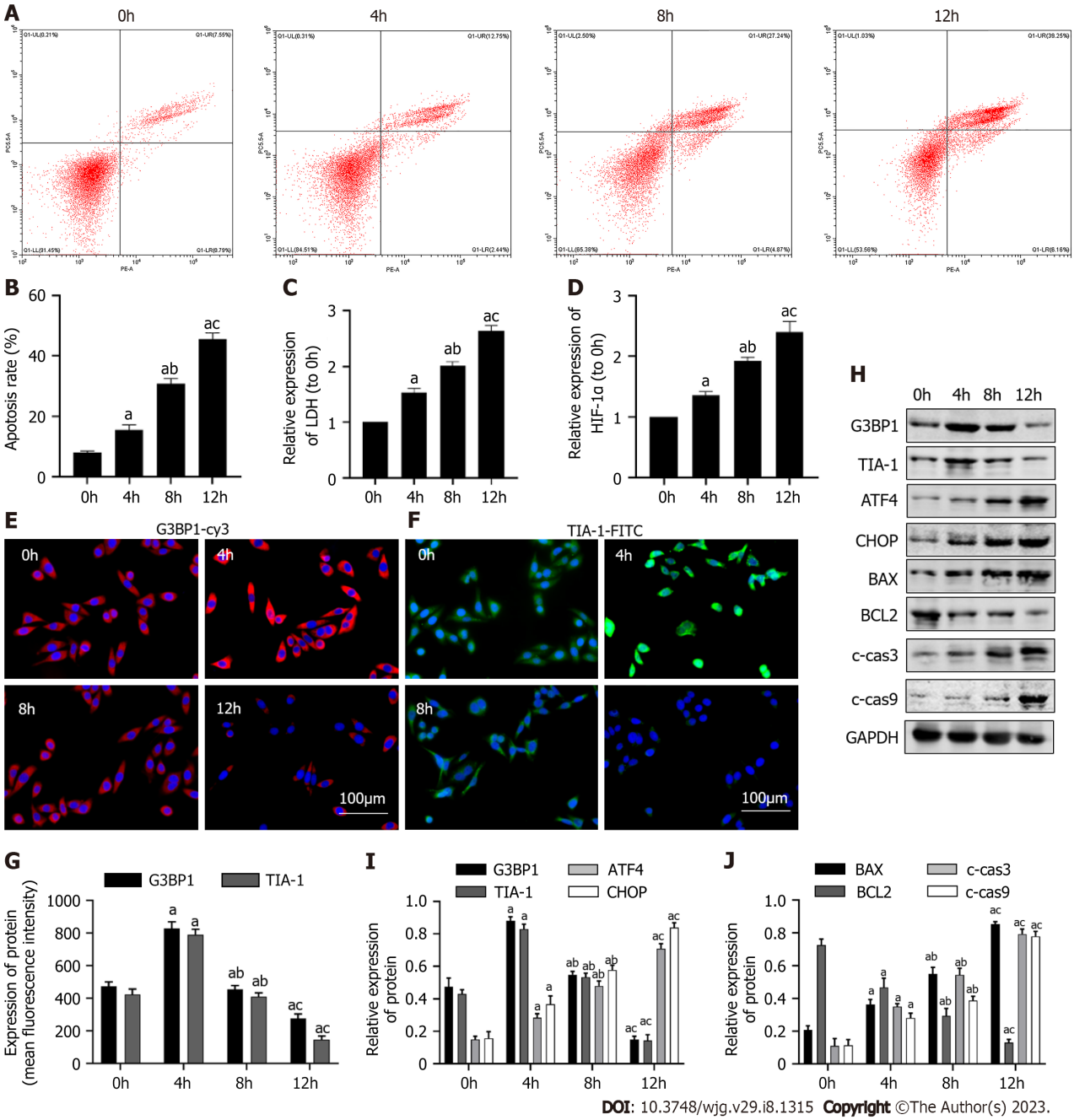

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Figure 1 Changes of stress granules, endoplasmic reticulum stress and apoptosis levels with the change of hypoxia time. A and B: The level of apoptosis was detected by flow cytometry; C and D: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant; E: The expression of G3BP1 in cells was detected by immunofluorescence; F: The expression of TIA-1 in cells was detected by immunofluorescence; G: The histogram displays the amount of molecules expression; H-J: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* 0 h group; $^{b}P < 0.05$ *vs* 4 h group; $^{c}P < 0.05$ *vs* 8 h group.

increased ($P < 0.05$). As shown in Figure 1E-G, compared with the normal group, the expression levels of G3BP1 and TIA-1, the marker molecules of SGs, were increased in the hypoxia group for 4 h ($P < 0.05$). The expression of G3BP1 decreased in the hypoxia groups of 8 h and 12 h compared with the hypoxia group of 4 h ($P < 0.05$). As shown in Figure 1H-J, compared with the normal group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the hypoxia group for 4 h increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was decreased ($P < 0.05$). After 8 h and 12 h hypoxia, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 were further increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was further decreased ($P < 0.05$). In conclusion, after 12 h hypoxia treatment, ERS and apoptosis levels of L02 cells were the highest, while SGs content was the lowest. Therefore, the cells treated with hypoxia for 12 h were used as cell models for subsequent research, which was consistent with reported studies[22].

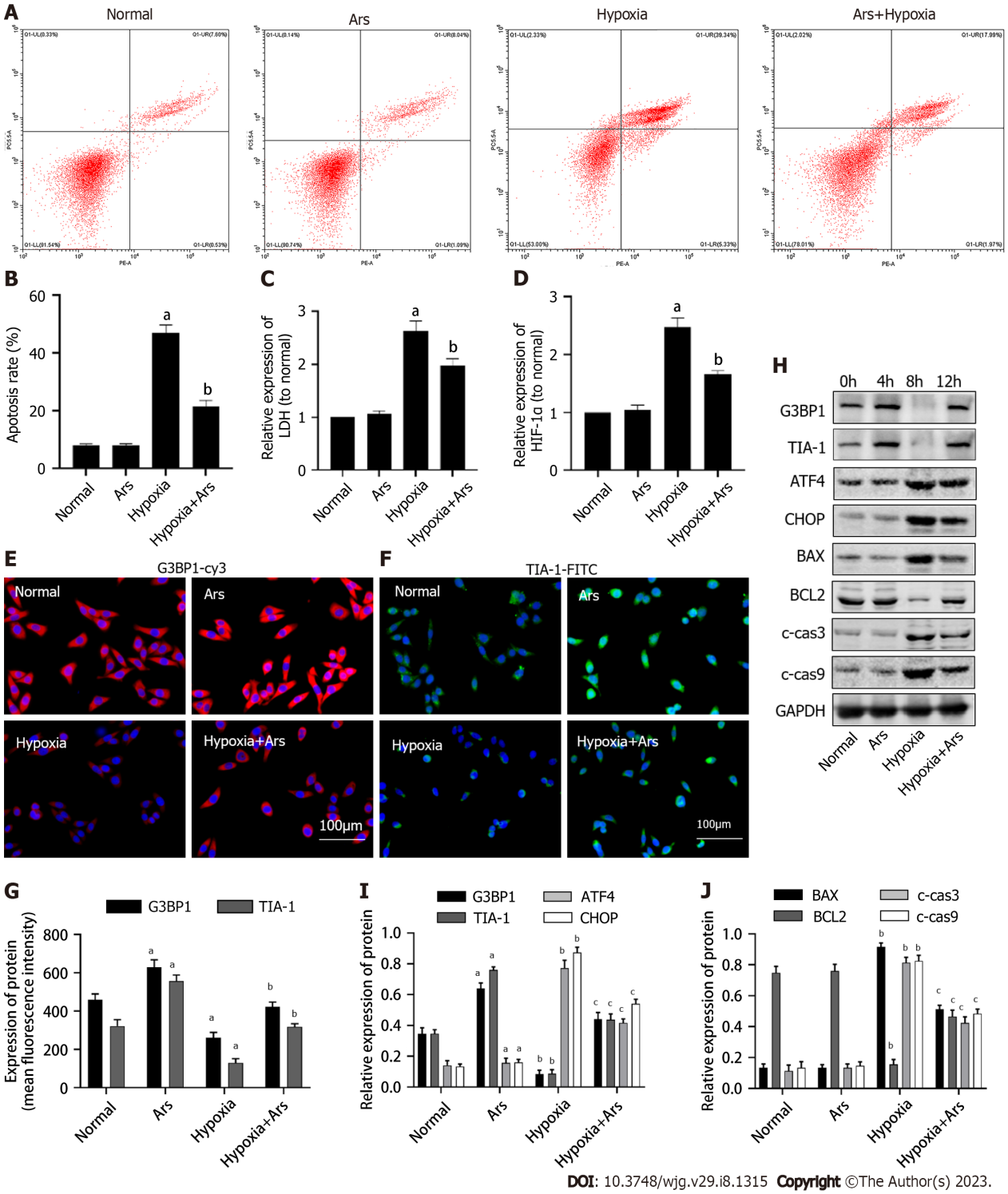

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Figure 2 Effects of stress granules agonist on endoplasmic reticulum stress and apoptosis in hepatocyte hypoxia model. A and B: The level of apoptosis was detected by flow cytometry; C and D: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant; E: The expression of G3BP1 in cells was detected by immunofluorescence; F: The expression of TIA-1 in cells was detected by immunofluorescence; G: The histogram displays the amount of molecules expression; H-J: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group.

Effects of SGs on ERS and apoptosis in hepatocyte hypoxia model

Arsenite can induce SGs formation by promoting e-IF2α phosphorylation[23], but arsenite has certain hepatotoxicity[24,25]. Refer to the reported studies, the 10 μmol/L intervention dose was used[25,26]. In this part, the Ars intervention group was set to observe that Ars could induce the formation of SGs without causing hepatocyte damage and related molecular changes.

As shown in Figure 2, we first verified the effect of SGs agonist Ars on normal L02 cells. Compared with the normal group, the expression level of G3BP1 was increased in the Ars group, while the level of

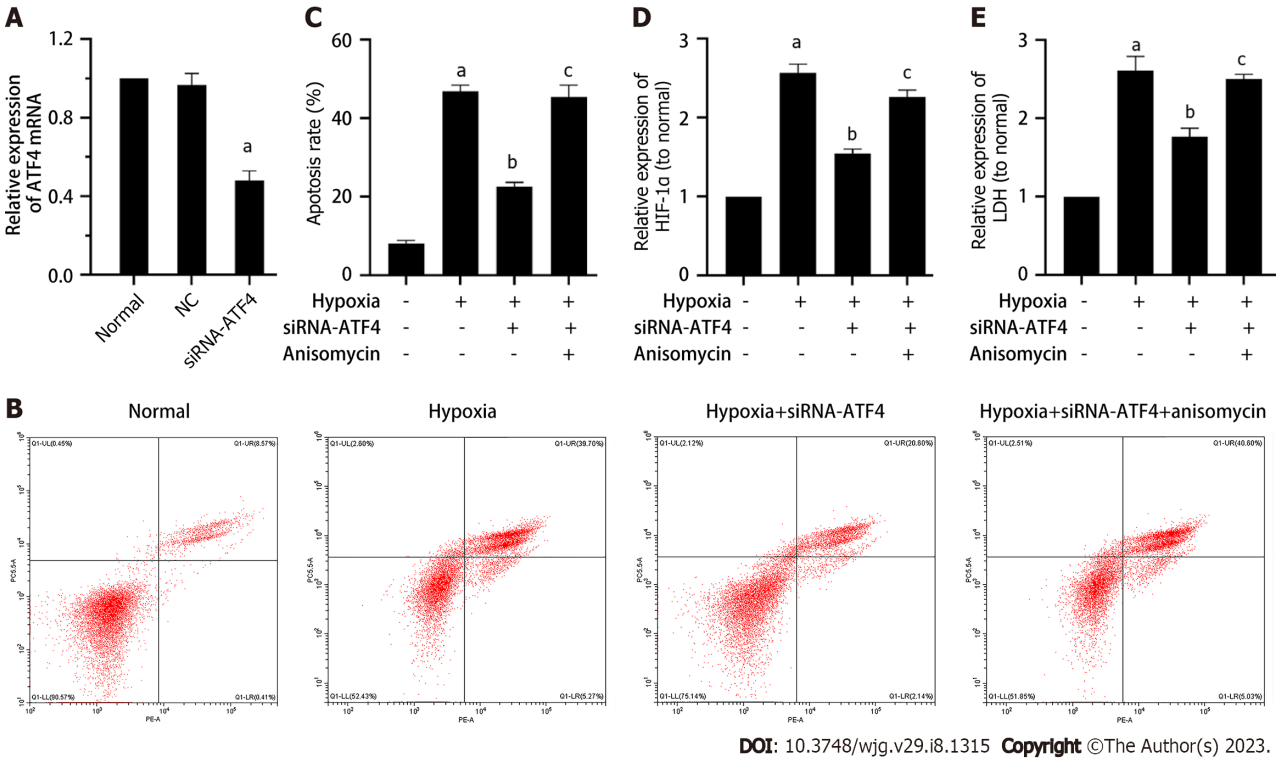

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Figure 3 Effect of stress granules on apoptosis, HIF-1α, and lactate dehydrogenase in hepatocyte hypoxia model through endoplasmic reticulum stress. A: The level of mRNA in cells was detected by quantitative real-time polymerase chain reaction; B and C: The level of apoptosis was detected by flow cytometry; D-E: The detection kits were used to evaluate the content of HIF-1α in homogenates, lactate dehydrogenase in cell supernatant. n = 3. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group; $^{c}P < 0.05$ *vs* hypoxia + siRNA-ATF4.

ERS and apoptosis did not change significantly. Subsequently, we used Ars to intervene the L02 hepatocyte model treated with 12 h hypoxia. As shown in Figure 2A-D, compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells in the Hypoxia + Ars group were decreased ($P < 0.05$). As shown in Figure 2E-G, compared with the Hypoxia group, the expression levels of G3BP1 and TIA-1 were increased in the Hypoxia + Ars group ($P < 0.05$). As shown in Figure 2H-J, compared with the Hypoxia group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + Ars group was decreased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was increased ($P < 0.05$).

Effect of SGs on apoptosis in hypoxia model through ERS

ATF4 plays a key role in the process of ERS, which negatively regulates Bcl-2 by promoting the expression of CHOP, leading to the transport of Bax from cytoplasm to mitochondria and the initiation of mitochondrial apoptosis pathway[27]. When excessive ERS occurs, PERK, IRE1, and ATF4 activate and activate the corresponding downstream factors, and up-regulate the expression of ATF4 at the transcriptional and translational levels, resulting in the destruction of the endoplasmic reticulum membrane, the efflux of Ca^{2+}, and the occurrence of apoptosis[28]. The knockdown of ATF4 and the SGs inhibitor anisomycin showed an antagonistic effect on the protective cells. In this part, the groups were set to show that SGs can affect hepatocyte injury through the ATF4-mediated ERS pathway.

We first verified the intervention effect of siRNA-ATF4. As shown in Figure 3A, compared with the normal group, the level of ATF4 mRNA in the normal control group did not change, while the level of ATF4 mRNA in the siRNA-ATF4 group decreased significantly ($P < 0.05$). As shown in Figure 3B-E, compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells of the Hypoxia + siRNA-ATF4 group were decreased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4, the apoptosis rate, HIF-1 α and LDH contents in cells of the Hypoxia + siRNA-ATF4 + anisomycin group were increased ($P < 0.05$). As shown in Figure 4A-C, compared with the Hypoxia group, the expression of G3BP1 and TIA-1 in Hypoxia + siRNA-ATF4 group was increased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4, the expression of G3BP1 and TIA-1 in Hypoxia + siRNA-ATF4 + anisomycin group was decreased ($P < 0.05$). As shown in Figure 4C-F, compared with the Hypoxia group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + siRNA-ATF4 group was decreased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was increased ($P < 0.05$). Compared with the Hypoxia + siRNA-ATF4 group, the protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Hypoxia + siRNA-ATF4 + anisomycin group was increased ($P < 0.05$), the expression of

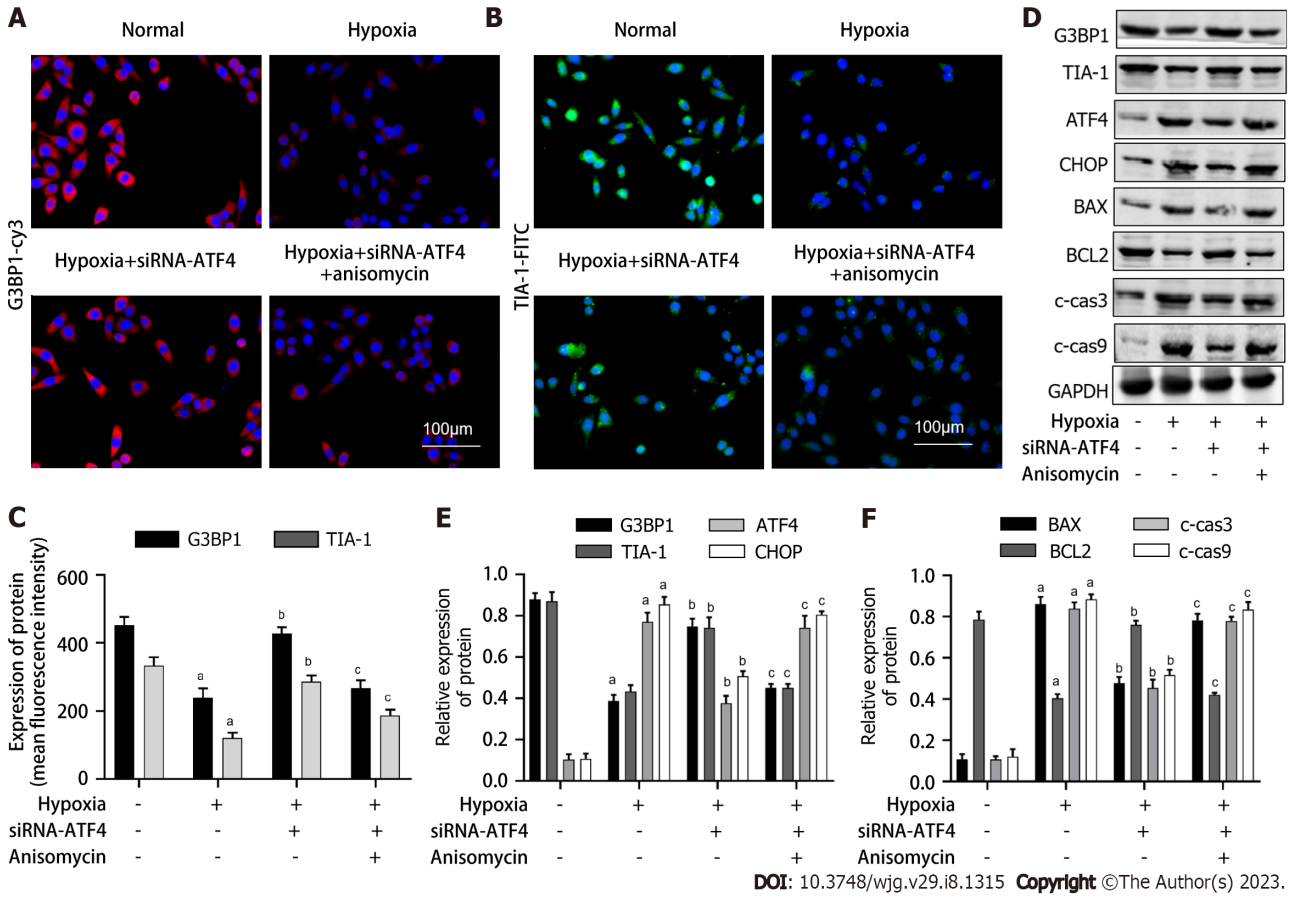

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Figure 4 Effect of stress granules on G3BP1 and endoplasmic reticulum stress related molecules in hepatocyte hypoxia model through endoplasmic reticulum stress. A: The expression of G3BP1 in cells was detected by immunofluorescence; B: The expression of TIA-1 in cells was detected by immunofluorescence; C: The histogram displays the amount of molecules expression; D-F: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. $n = 3$. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* hypoxia group; $^{c}P < 0.05$ *vs* hypoxia + siRNA-ATF4.

BCL2 was decreased ($P < 0.05$).

Effects of SGs on ERS and apoptosis levels in liver of ALF mice

In the *in vivo* experiment, we first detected whether the ALF mouse model was successfully established, and then detected the effects of Ars on liver pathological changes and serum biochemical indexes. As shown in Figure 5A, the liver lobules in the normal group were clearly structured and the hepatocytes were neatly arranged. The lobule structure of liver tissue in ALF group was not clear, hepatocyte was necrotic around with inflammatory cells infiltrate. Compared with ALF model group, the hepatic lobule structure in Ars group was clearer and the infiltration of inflammatory cells was reduced. As shown in Figure 5B and F, compared with the normal group, the apoptosis level in liver tissues of mice in model group was significantly increased ($P < 0.05$). After Ars intervention, apoptosis level of liver tissue was significantly reduced ($P < 0.05$). As shown in Figure 5C-E, the serum levels of ALT, AST and TBIL in model group were higher than those in normal group ($P < 0.05$). Compared with model group, ALT, AST and TBIL levels in Ars group were significantly decreased ($P < 0.05$). In addition, as shown in Figure 5G, the 24 h survival rate of mice was observed in each group. The results showed that 90% of mice survived in Ars group, whereas only 70.0% in model group.

As shown in Figure 6A and B, compared with the normal group, the expression of G3BP1 and TIA-1 in model group was decreased ($P < 0.05$). Compared with the model group, the expression of G3BP1 and TIA-1 in Ars group was increased ($P < 0.05$). As shown in Figure 6C-G, compared with the normal group, the level of HIF-1α and LDH, the protein expression of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the model was increased ($P < 0.05$), the expression of G3BP1, TIA-1, BCL2 was decreased ($P < 0.05$). Compared with the model group, the level of HIF-1α and LDH, the protein expression of protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Ars group was decreased ($P < 0.05$), the expression of BCL2 was increased ($P < 0.05$).

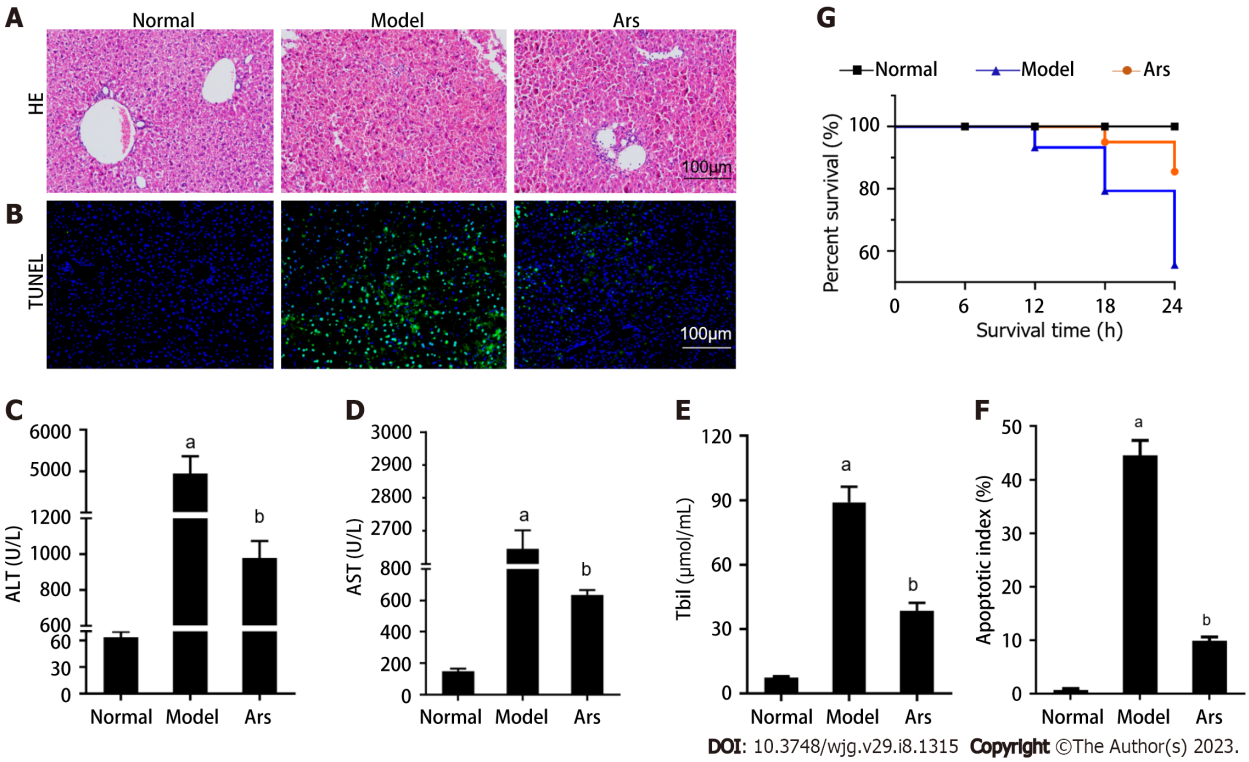

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Figure 5 Effects of stress granules agonist on histopathology, apoptosis levels, liver function in acute liver failure mice. A: Hepatic histopathological changes were detected by HE staining; B and F: TUNEL stanning was used to detected the level of apoptosis in live liver tissue; C-E: The levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin in the serum of mice were detected by automatic biochemical analyzer; G: The 6 h, 12 h, 18 h, 24 h survival rates of mice in each group were observed. n = 10. Data was shown mean ± SD. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* model group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin.

DISCUSSION

Liver is very sensitive to ischemic hypoxic injury because of its high aerobic tissue and metabolism. Ischemia-hypoxia and IR injury induce a large number of ERS and mitochondrial apoptosis pathway. ERS can be induced under ischemia and hypoxia through HIF-1α[29,30]. The complex functions of ERS in hepatocytes can regulate inflammation, oxidative stress, *etc*., which are closely related to the pathogenesis of liver failure. A large number of experimental studies have confirmed that ERS occurs in the liver of various types of ALF animal models. In the liver of ALF mice/rats induced by N-acetaminophen (APAP)[31], LPS combined with D-Gal[32], and carbon tetrachloride[33] induced liver, p-eIF2α, ATF4 and CHOP GRP78 markedly increased. The ERS inhibitors 4-phenylbutyric acid[34] and TUDCA[35] can alleviate the up-regulation of endoplasmic reticulum stress markers CHOP, XBP1, p-eIF2α, and reduce inflammation in liver tissue. damage, alleviate the up-regulation of serum transaminases. At the same time, inhibiting of ERS had a protective effect on APAP-induced liver injury, can reduce liver necrosis, and improve mouse survival[36]. It can be seen that ERS-related molecules can be used as new targets, and the development of drugs targeting ERS provides new ideas for the treatment of ALF.

ERS is an important homeostatic device during liver disease progression, which can assist cells to resist stress, but excessive stress can also lead to cell damage. In the early stage of stress, the endoplasmic reticulum reduces the cell load caused by the accumulation of wrong proteins by inhibiting protein synthesis and accelerating protein transport and degradation, and maintains cell stability[37]. However, when the stress intensity continues to increase, ERS will initiate cell apoptosis pathway. Among them, ATF4 induces the production of endoplasmic reticulum oxide protein and activates the inositol triphosphate receptor, which is a key way to induce apoptosis[38].

SGs are formed by RNA and protein aggregates that control RNA metabolism, signaling, and cell survival under stress. When translation is inhibited, polysomes lose their mRNAs, and "naked" mRNAs assemble with SG nucleating proteins for liquid-liquid phase separation[39]. SGs are dynamically complex and variable biomolecular condensates whose composition and structure undergo dramatic changes under different types of stress. The ability of cells to respond rapidly to various environmental stresses[40,41]. Therefore, the dynamic process of SGs and their regulation are crucial for cells to cope with stress. Studies have shown that the process of SGs formation is stalled by the accumulation of translation initiation complexes in response to various stresses[42]. G3BP1 bind mRNA and aggregate to form SGs. While large SGs aggregates are formed by smaller aggregates through post-translational

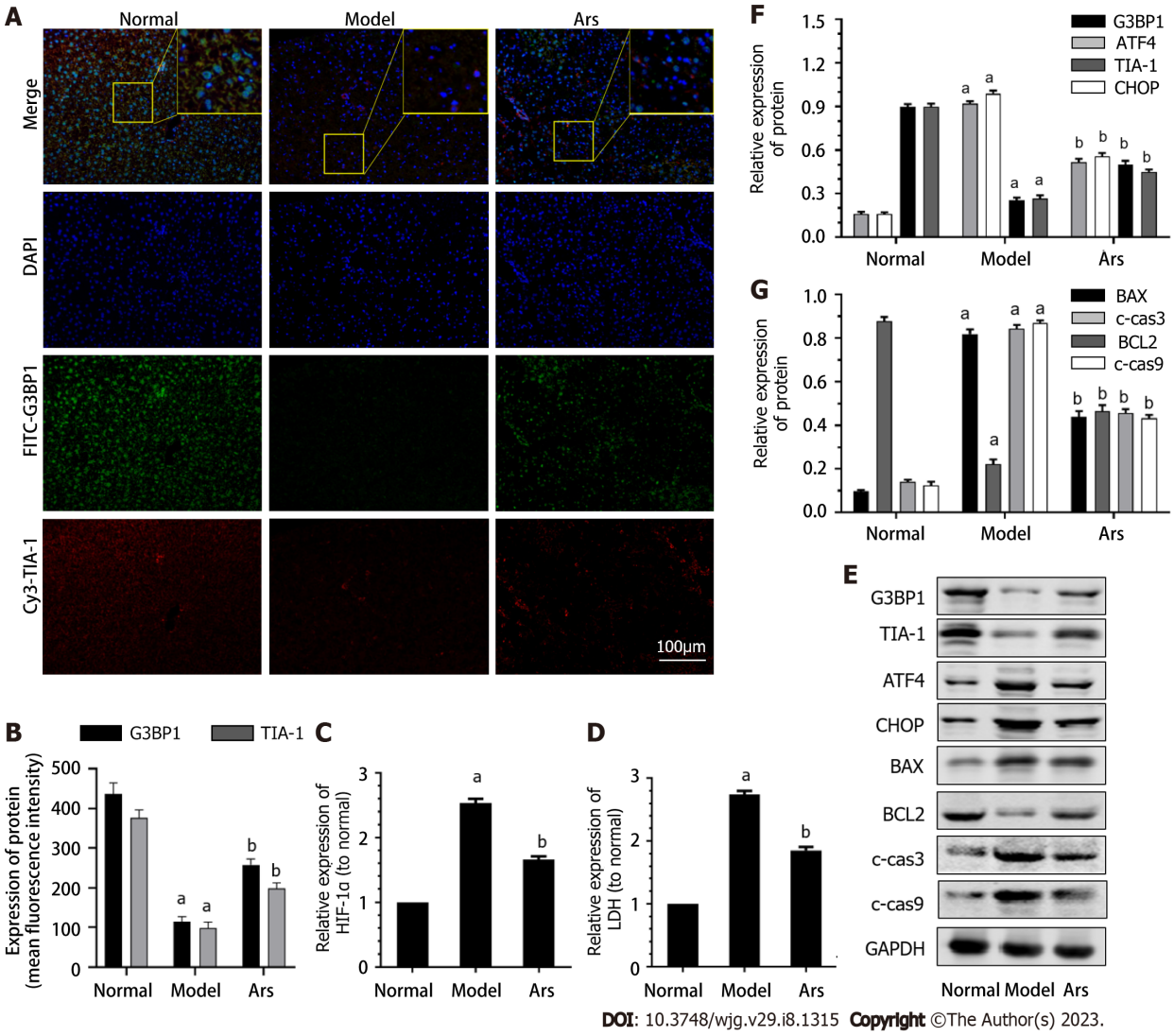

Figure 6 Effect of stress granules agonist on G3BP1, HIF-1α, lactate dehydrogenase and endoplasmic reticulum stress related molecules in acute liver failure mice liver. A: The expression of G3BP1 and TIA-1 in liver was detected by immunofluorescence; B: The histogram displays the amount of molecules expression; C and D: The detection kits were used to evaluate the content of HIF-1α in liver tissue homogenate, lactate dehydrogenase in serum; E-G: The protein expression levels of G3BP1, TIA-1, ATF4, CHOP, BAX, BCL2, c-cas3 and c-cas9 was detected by western blot. n = 10. Data was shown mean ± SD. ^{a}P < 0.05 *vs* normal group; ^{b}P < 0.05 *vs* model group.

modification and microtubule transport[43]. Another typical SGs maker, TIA-1 RNA-binding protein packet TIA-1 binds to mRNA, causing mRNA to stop translation and aggregate to form SGs[44,45]. Therefore, G3BP1 and TIA-1 were selected as markers for SGs in this study. Therefore, G3BP1 and TIA-1 were selected as markers for SGs in this study.

There is no relevant report on the relationship between SGs, ERS and hepatocyte apoptosis in the process of hepatocyte ischemia and hypoxia for ALF. In the *in vitro* study, we firstly observed how the levels of SGs, ERS and apoptosis changed over time. With the prolongation of hypoxia time, the levels of ERS and apoptosis in hepatocytes increased. The level of SGs increased at 4h and then decreased. Therefore, hepatocyte treated with hypoxia for 12 h were used as cell models for subsequent research. This suggested that SGs were stress-increased in the early stage of hepatocyte hypoxia to protect cells from damage. However, with the prolongation of hypoxia time, the production of SGs would decrease, and the effect of protecting hepatocytes will be weakened. Then the effects of SGs on ERS and apoptosis in hepatocyte hypoxia model was observed. Compared with the Hypoxia group, the apoptosis rate and ERS level decreased in SGs hepatocyte Ars treated group. However, Ars could elevate the level of SGs. In the next, it was verified the effect of SGs on apoptosis level of hepatocyte hypoxia model through ERS. It was verified the intervention effect of siRNA-ATF4. Compared with the normal group, the level of ATF4 mRNA in the siRNA-ATF4 group was decreased significantly. Compared with the Hypoxia group, the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased in the siRNA-ATF4 treatment group. Moreover, on the basis of siRNA-ATF4 intervention group, it was found that the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased.

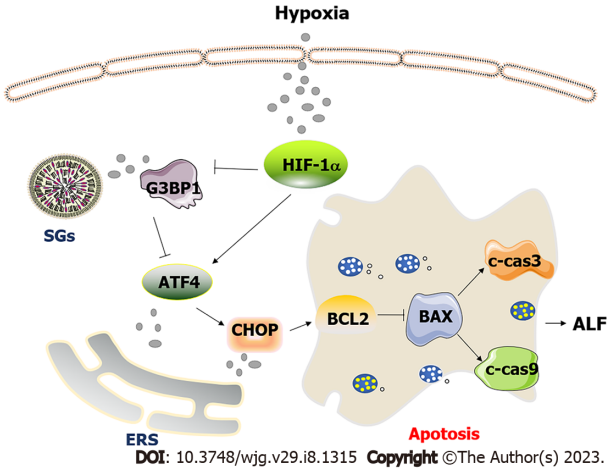

Figure 7 Graphical abstract. In the process of acute liver failure, hepatocytes were damaged by hypoxia and ischemia injury. At this time, the content of HIF-1α in cells increased, which inhibited the formation of stress granules (SGs) mediated by G3BP1 and promoted the expression of endoplasmic reticulum stress (ERS) marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway. SGs: Stress granules; ERS: Endoplasmic reticulum stress; ALF: Acute liver failure.

According to previous studies, the criteria for success in animal modeling of ALF include liver histopathological damage, serological changes, especially elevated levels of transaminase and bilirubin [13,16,46]. In this study, LPS injection combined with D-Gal simulates acute inflammatory liver injury model, which is widely accepted and used to explore and develop new liver protective reagents for inflammatory liver injury[47]. LPS is in the outer membrane of the gram-negative bacteria[48], and can combine with myeloid differentiation factor 2 and cluster of differentiation 14 to form a complex. This complex is recognized by the toll-like receptor 4 on the membrane of Kupffer cells (KCs) in the liver. KCs can produce the inflammatory mediators, such as TNF-α, IL-1β, IL-6, *etc.*, leading to liver cell damage[49,50]. However, LPS has a low specificity for liver injury, so it is often combined with DGal to establish an animal model of acute inflammatory liver injury to simulate human hepatitis[48]. Studies have shown that D-Gal can inhibit protein synthesis by depletion of uridine triphosphate through the galactose pathway, and reactive oxygen species production can induce liver injury. Therefore, D-Gal can be used as a sensitizer in LPS-induced liver injury[51]. In the course of occurrence and development of liver failure, a large number of liver cell death is the most core event.

So, In the *in vivo* experiment, it was first detected whether the ALF mouse model was successfully established. HE staining showed that the liver tissue structure of mice in the normal group was regular, and the liver cells were neatly arranged in the hepatic lobules. No necrosis of liver cells and inflammatory cell infiltration. The liver lobule structure of the ALF model group was destroyed by LPS combined with D-Gal, accompanied by a large number of necrotic liver cells and infiltrating inflammatory cells. Compared with normal group, ALT, AST and TBIL levels in serum of model group were increased. The above studies indicate that the mouse model of ALF has been successfully constructed in this study, which is consistent with previous reports[13,16]. After Ars intervention, the degree of infiltration of necrotic liver cells and inflammatory cells in mouse liver tissue was reduced, and the levels of ALT, AST and TBIL in serum were decreased.

Moreover, compared with the normal group, the apoptosis level in liver tissues of mice in model group was significantly increased. The expression of G3BP1 in model group was decreased. The expression of G3BP1 in model group was decreased. Moreover, compared with the normal group, the level of HIF-1α and LDH, the protein expression of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the model was increased, the expression of BCL2 was decreased. Compared with the model group, the level of HIF-1α and LDH, the protein expression of protein expression levels of ATF4, CHOP, BAX, c-cas3 and c-cas9 in the Ars group was decreased, the expression of BCL2 was increased.

CONCLUSION

In conclusion, as shown in Figure 7, hepatocytes were damaged by hypoxia and ischemia injury in the process of ALF. At this time, the content of HIF-1α in cells increased, which inhibited the formation of SGs mediated by G3BP1 and promoted the expression of ERS marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway. However, the inhibition of ERS-mediated hepatocyte apoptosis pathway by SGs still needs to be further studied. Despite the use of Ars and anisomycin in the intervention of SGs, there is still no direct evidence to prove the influence of

SGs on hepatocyte apoptosis. In the next studies, we will use more appropriate methods to directly detect the influence of SGs on hepatocyte. At the same time, this paper provides potential targets and ideas for clinical treatment of ALF.

ARTICLE HIGHLIGHTS

Research background

There is no relevant report on the relationship between stress granules (SGs), endoplasmic reticulum stress (ERS) and hepatocyte apoptosis in the process of hepatocyte ischemia and hypoxia for acute liver failure (ALF).

Research motivation

This paper provides potential targets and ideas for clinical treatment of ALF.

Research objectives

This study was to investigate whether SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS mediated apoptosis.

Research methods

The agonist of SGs, arsenite (Ars) was used to intervene hypoxia-induced hepatocyte injury cellular model and ALF mice models. Further, the siRNA of ATF4 and SGs inhibitor anisomycin was then used to intervene in cell models.

Research results

In the *in vitro* study, we firstly observed how the levels of SGs, ERS and apoptosis changed over time. With the prolongation of hypoxia time, the levels of ERS and apoptosis in hepatocytes increased. The level of SGs increased at 4h and then decreased. Therefore, hepatocyte treated with hypoxia for 12 h were used as cell models for subsequent research. This suggested that SGs were stress-increased in the early stage of hepatocyte hypoxia to protect cells from damage. However, with the prolongation of hypoxia time, the production of SGs would decrease, and the effect of protecting hepatocytes will be weakened. Then the effects of SGs on ERS and apoptosis in hepatocyte hypoxia model was observed. Compared with the Hypoxia group, the apoptosis rate and ERS level decreased in SGs hepatocyte Ars treated group. However, Ars could elevate the level of SGs. In the next, it was verified the effect of SGs on apoptosis level of hepatocyte hypoxia model through ERS. It was verified the intervention effect of siRNA-ATF4. Compared with the normal group, the level of ATF4 mRNA in the siRNA-ATF4 group was decreased significantly. Compared with the Hypoxia group, the apoptosis rate, HIF-1α and lactate dehydrogenase (LDH) contents in cells, the level of ERS was decreased in the siRNA-ATF4 treatment group. Moreover, on the basis of siRNA-ATF4 intervention group, it was found that the apoptosis rate, HIF-1α and LDH contents in cells, the level of ERS was decreased. In the *in vivo* experiment, it was first detected whether the ALF mouse model was successfully established. HE staining showed that the liver tissue structure of mice in the normal group was regular, and the liver cells were neatly arranged in the hepatic lobules. No necrosis of liver cells and inflammatory cell infiltration. The liver lobule structure of the ALF model group was destroyed by LPS combined with D-Gal, accompanied by a large number of necrotic liver cells and infiltrating inflammatory cells. Compared with normal group, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) levels in serum of model group were increased. The above studies indicate that the mouse model of ALF has been successfully constructed in this study, which is consistent with previous reports. After Ars intervention, the degree of infiltration of necrotic liver cells and inflammatory cells in mouse liver tissue was reduced, and the levels of ALT, AST and TBIL in serum were decreased.

Research conclusions

Hepatocytes were damaged by hypoxia and ischemia injury in the process of ALF. At this time, the content of HIF-1α in cells increased, which inhibited the formation of SGs mediated by G3BP1 and promoted the expression of ERS marker molecules ATF4 and CHOP. The activated ERS pathway further promotes hepatocyte apoptosis. Promoting SGs synthesis can inhibit the level of hepatocyte apoptosis by inhibiting the ATF4-mediated ERS pathway.

Research perspectives

SGs could protect hepatocytes from hypoxia-induced damage during ALF by reducing ERS-mediated apoptosis.

FOOTNOTES

Author contributions: Wang Y takes responsibility for the integrity of the work as a whole, from inception to published article; Wang Y and Li WY conceived and designed the study; Li WY, Yang F, Li X and Wang LW perform the experiment; Li WY and Wang Y wrote the paper; Wang Y edited the article; all authors approved the final version of the manuscript.

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ORCID number: Xun Li 0000-0002-9598-8984; Lu-Wen Wang 0000-0002-0654-1196; Yao Wang 0000-0001-7701-5313.

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ORIGINAL ARTICLE

Observational Study

Intestinal complications in Brazilian patients with ulcerative colitis treated with conventional therapy between 2011 and 2020

Adalberta Lima Martins, Rodrigo Galhardi Gasparini, Ligia Yukie Sassaki, Rogerio Saad-Hossne, Alessandra Mileni Versut Ritter, Tania Biatti Barreto, Taciana Marcolino, Claudia Yang Santos

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Adalberta Lima Martins, Espirito Santo Health Office, State Office for Pharmaceutical Assistance, Espirito Santos 29056-030, Brazil

Rodrigo Galhardi Gasparini, Department of Gastroenterology, Specialized Medical Center, Marília 17502-020, Brazil

Ligia Yukie Sassaki, Rogerio Saad-Hossne, Department of Gastroenterology, Sao Paulo State University, Medical School, Botucatu 18618-687, Brazil

Alessandra Mileni Versut Ritter, Real World Evidence, IQVIA Brazil, Sao Paulo 04719-002, Brazil

Tania Biatti Barreto, Taciana Marcolino, Claudia Yang Santos, Department of Gastroenterology, Takeda Pharmaceuticals Brazil, Sao Paulo 04794-000, Brazil

Corresponding author: Claudia Yang Santos, BPharm, MSc, PhD, Research Scientist, Department of Gastroenterology, Takeda Pharmaceuticals Brazil, Av. das Nações Unidas, 14401 Torre Jequitiba, 11 Andar, Sao Paulo 04794-000, Brazil. clausantos2910@gmail.com

Abstract

BACKGROUND

This was an observational, descriptive, and retrospective study from 2011 to 2020 from the Department of Informatics of the Brazilian Healthcare System database.

AIM

To describe the intestinal complications (IC) of patients with ulcerative colitis (UC) who started conventional therapies in Brazil´s public Healthcare system.

METHODS

Patients ≥ 18 years of age who had at least one claim related to UC 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) code and at least 2 claims for conventional therapies were included. IC was defined as at least one claim of: UC-related hospitalization, procedures code for rectum or intestinal surgeries, and/or associated disease defined by ICD-10 codes (malignant neoplasia of colon, stenosis, hemorrhage, ulcer and other rectum or anus disease, megacolon, functional diarrhea volvulus, intussusception and erythema nodosum). Descriptive statistics, annual incidence, and

incidence rate (IR) [per 100 patient-years (PY)] over the available follow-up period were calculated.

RESULTS

In total, 41229 UC patients were included (median age, 48 years; 65% women) and the median (interquartile range) follow-up period was 3.3 (1.8-5.3) years. Conventional therapy used during follow-up period included: mesalazine (87%), sulfasalazine (15%), azathioprine (16%) or methotrexate (1%) with a median duration of 1.9 (0.8-4.0) years. Overall IR of IC was 3.2 cases per 100 PY. Among the IC claims, 54% were related to associated diseases, 20% to procedures and 26% to hospitalizations. The overall annual incidence of IC was 2.9%, 2.6% and 2.5% in the first, second and third year after the first claim for therapy (index date), respectively. Over the first 3 years, the annual IR of UC-related hospitalizations ranged from 0.8% to 1.1%; associated diseases from 0.9% to 1.2% - in which anus or rectum disease, and malignant neoplasia of colon were the most frequently reported; and procedure events from 0.6% to 0.7%, being intestinal resection and polyp removal the most frequent ones.

CONCLUSION

Study shows that UC patients under conventional therapy seem to present progression of disease developing some IC, which may have a negative impact on patients and the burden on the health system.

Key Words: Ulcerative colitis; Brazil; Conventional therapy; Intestinal complications; Real world; Public healthcare

Core Tip: This population-based study investigated intestinal complications (ICs) in patients with ulcerative colitis (UC) undergoing therapy available in the public healthcare system (Sistema Único de Saúde) of Brazil over the last decade. Our results showed that some patients with UC undergoing conventional therapy, seem to present an active and progressive disease and develop relevant ICs, which demand important resources of the healthcare system and have a negative impact on their lives.

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INTRODUCTION

Ulcerative Colitis (UC) is an idiopathic inflammatory disease of the mucosa and colon that commonly involve colonic regions both proximal to and distal from the site of the primary lesion[1]. The typical presentation includes symptoms such as bloody diarrhea, rectal urgency, and abdominal pain[2]. UC is a component of inflammatory bowel diseases (IBD), which are chronic, immune-mediated inflammatory diseases of the gastrointestinal tract. The etiopathogenesis is multifactorial involving different environmental, genetic, immune-mediated and intestinal microbial factors[3].

Although epidemiological studies are still scarce in developing countries, the incidence and prevalence of UC is increasing worldwide, along with IBD[4]. A systematic review showed that the incidence and prevalence of UC in Latin America varied between regions and studies, ranging from 0.04 to 8.00/100000 and 0.23 to 76.1/100000, respectively, and generally increased over the period from 1986 to 2015[5].

Many patients have long periods of complete remission, but the cumulative probability of remaining relapse-free for two years is only 20%, decreasing to less than 5% in 10 years[6]. Chronic inflammation of the colorectal mucosa, as is typically found in UC, is a proven risk factor for neoplasm of colon. The development of neoplasm of colon in patients with UC is related to several factors: Duration, extent, activity, severity and family history of the disease. The progression from UC inflammation to neoplasm and/or surgery is a multi-step process in which the accumulation of symptoms and sequential mucosal changes gradually transition to a low-grade change to neoplasm[7]. Indications for emergency surgery include refractory toxic megacolon, perforation, and ongoing severe colorectal bleeding[8].

UC is a progressive disease that can lead to other serious complications and/or even life-threatening complications. One of the most common UC disease progressions are: anorectal dysfunction (50%)[9], proximal extension (25%) with the greatest proximal extension occurring during the first 10 years[10, 11], pseudopolyposis (16%)[12] and other such as impaired permeability in remission or with mucosal healing, stenosis and dysmotility[10]. Thus, complications related to UC represent a substantial burden in terms of costs and patient quality of life, with hospital admissions, operations and treatments being the most relevant burdens[13].

The treatment of UC currently aims to monitor indicators of disease activity and to adjust therapy, aiming to achieve clinical remission and prevent long-term complications, such as dysplasia, colorectal cancer, hospitalizations and colectomy[14]. In 2021, the STRIDE-II study confirmed STRIDE-I's long-term goals of clinical remission, histological remission, and endoscopic mucosal healing[15] and added absence of disability, restoration of quality of life, and normal growth in children. Symptomatic relief and normalization of serum and fecal markers were determined as short-term targets[16].

Treatment will vary depending on the severity and location of the UC. For proctitis, topical therapy with 5-aminosalicylic acid (5-ASA) compounds is used. For more severe UC, oral and local 5-ASA compounds and corticosteroids are indicated to induce remission. For patients who do not respond to these treatments, intravenous steroids are used. When refractory to this, calcineurin inhibitors (cyclosporine, tacrolimus), tumor necrosis factor-α antibodies or immunomodulators are then used[17].

For patients with moderate to severe UC, drugs such as thiopurines and biologics are indicated to control symptoms and heal the intestinal mucosa. Anti-tumor necrotizing factor (TNF) drugs include infliximab, adalimumab and golimumab. Of the anti-integrin drug, vedolizumab can be mentioned. And the anti-p40 antibody targeting interleukin (IL)-12 and IL-23, we can mention ustekinumab and small molecules (Janus Kinase inhibitor - tofacitinib)[18]. Biologics were not available in the public healthcare system during the study period in Brazil. Studies have observed an association between the administration of biologics therapies such as anti-TNF-α within the first 2 years of diagnosis and several beneficial effects, including reduction risk of intestinal surgery[4,5,19].

There are few population-based studies documenting the evaluation and progression of the conventional therapy-using patient of the UC disease phenotype. Thus, this study aims to describe the intestinal complications (IC) of patients with UC who started treatment with conventional therapies between January 2011 and January 2020 in the Brazilian healthcare system.

MATERIALS AND METHODS

Data source

For this study, data were collected from two Department of Informatics of the Brazilian Healthcare System (DATASUS) databases: Hospitalizations Information System (SIH), which contains hospitalization data[20] and the Outpatient Information System (SIA), which contains data on outpatient care [21,22].

The causes of hospital admissions are coded according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). The SIH was used to identify the number and causes of hospital admissions related to UC and/or its IC.

The SIA is the system that allows local managers to process outpatient care information recorded in outpatient care capture applications by public and private providers contracted/agreed by the public healthcare system (SUS). Most SIA subsets have an ID code that enables linking the outpatient procedures to a single patient. Because SIH does not have patient ID information, a probabilistic record linkage (RLK) was performed allowing longitudinal assessment in SIH and SIA at a patient-level. The RLK relied on multiple steps with different combination of patient information from both databases, such as date of birth, gender and ZIP code, making it possible to identify patients in both systems.

Data is presented as procedure codes from billing records and includes demographic information, all procedures such as inpatient and outpatient performance, number of procedures, and other additional information.

Study design

The present study is a descriptive, observational, retrospective study of secondary data collected between 2011 and 2020, which used the DATASUS to characterize IC in patients with UC.

We considered UC patients those who had at least one report of UC ICD-10 code (K51). The date of the first request for UC conventional therapy was considered as the index date. Conventional therapies used for UC treatment were based on procedures codes available at DATASUS, according to the following list: Sulfasalazine; Mesalazine; Azathioprine; Methotrexate; Cyclosporine. UC patients must had at least two claims of conventional therapy to be included in the study. They were followed from the index date until the last information available in the DATASUS regardless of being under conventional treatment or not. One or more conventional therapies could had been used at the same time and/or in sequence by the patient, due to that line of treatment and comparisons between different conventional therapies were not assessed in this study.

We also assessed sociodemographic, such as age, sex and location of residency of the patient. Measurements of IC information in UC patients were extracted, including procedures performed, hospitalization causes, and diseases developing in the study sample. Each pre-defined claim of IC reported after conventional therapy initiation was considered as a progression of the UC disease.

Pre-defined claims of ICs were validated based on four independent medical expert opinions and literature about the natural history and evaluation of UC[23]. Patients who progressed from UC to malignant neoplasm of colon, stenosis, hemorrhage, ulcer or other specified diseases or symptoms of anus and rectum, functional diarrhea, megacolon, volvulus, intussusception or erythema nodosum or any intestinal surgery or hospitalization related to the disease was considered to have experienced an IC.

The following claims were considered as proxy of ICs: any claim for anus, rectum or intestinal surgeries for procedures, any claim of hospitalization reporting UC ICD-10 codes for hospitalization and/or any claim of ICD-10 code pre-defined for associated diseases [Table 1, list of diseases (ICD-10 code) considered as IC in consequence of underlying UC disease].

Overall ICs could reflect one or more type of ICs, so, in an attempt to represent them, IC analyses sets were stratified into 3 groups based on the type of IC: (1) Procedures: If claim of procedure code for anus, rectum or intestinal surgeries; (2) Hospitalizations: If claim of hospitalization for UC (reported UC ICD-10 in inpatient setting); and (3) Associated disease: If claim of ICD-10 code for diseases pre-defined as UC complications, such as C18 (malignant neoplasm of colon), K62.4 (stenosis of anus and rectum), K62.5 (hemorrhage of anus and rectum), K62.6 (ulcer of anus and rectum), K62.8 (other specified diseases of anus and rectum), K62.9 (disease of anus and rectum, unspecified), K59.1 (functional diarrhea), K59.3 (megacolon, not elsewhere classified), K56.2 (volvulus), K56.1 (intussusception), or L52 (erythema nodosum). Thus, to be considered a case of IC, patients must have at least one medical complaint in one or more of the sub-groups of ICs previously described.

In the data analysis, the annual IC in UC patients per each calendar year, and the incidence per 100 people of ICs were evaluated throughout the study. Patients whose UC progressed after the conventional treatment initiation timeframe were recorded as an IC annual rate and expressed year (from 1 to 5 years) according to the number of claims (total and mean) and the number of patients (total and percentage according to the number of patients in follow-up at the database at the respective year Also, the mean ICs per each patient were assessed and expressed by calendar year. The incidence of IC was converted into units per patient/year by dividing them for all patients by the person-years of follow-up, because each individual attends a different time in the database. Thus, the incidence rate was calculated as the number of intestinal events divided by the total person-time at risk. Considering that one patient could present one or more types of IC, we also expressed the total of ICs found in the study.

The most frequent ICs related to procedures in UC population were assessed according to the number of patients with at least one procedure for anus, rectum or intestinal surgeries during the study period. The most frequent ICs related to associated disease in UC population was assessed according to the number of patients with at least one ICD-10 code of pre-defined associated-disease.

Study population

Patients were included in the study if they were aged ≥ 18 years old, if they had evidence of UC [at least one ICD-10 claim of UC (K51.0, K51.2, K51.3, K51.4, K51.5, K51.8 or K51.9)] and if they had at least one claim of conventional treatment of UC. The UC conventional therapies (synthetic) considered in the present study were sulfasalazine, mesalazine, azathioprine, methotrexate and cyclosporine.

To capture the initial stage of treatment, patients must have had no claims for conventional and/or anti-TNF therapy prior to the index date. Thus, the period from January 2010 to December 2011 was considered only for the evaluation of the medical history of patients who had the index date in 2011.

Considering that fissure and fistula of anal and rectal regions (K60) and/or abscess of anal and rectal regions (K61) are considered an uncommon IC in UC patients, but common in other IBD, we excluded patients with at least one ICD-10 claim of these diseases after index date (first claim of UC conventional treatment code). Additionally, patients with less than 6 months of follow-up in the database and patients who were not exclusive to the SUS were excluded. Non-SUS exclusive was defined as patients who used the SUS only to obtain high-cost medications and who carried out the rest of the treatment and medical care through their private health plan[24]. Thus, they are patients who had claims related to medications only.

Statistical analysis

Although only descriptive analyses were performed, a statistical review of the study was performed by a statistician. Results were described as measures of central tendency (means, medians) and spread (variance, range) for continuous variables (*e.g.*, age); absolute number and percentage for categorical variables (*e.g.*, sex) and Kaplan Meier Curve for time to event data. Ninety-five percent confidence intervals (95%CI) were calculated according to the variable, normal distribution for continuous variable, normal approximation to binominal distribution for proportion and Poisson distribution for incidence rate, as applicable.

Table 1 Description of most common intestinal complications related to associated diseases (International Statistical Classification of Diseases and Related Health Problems) or procedures (codes), *n* (%)

ICs	Description of ICs	Patients (*n* = 1273)	%	Procedure-related complications		Patients (*n* = 1125)	%
Associated disease (ICD-10–related complications)				0407020403	Abdominal rectosig-moidectomy	167	15
K62	Other diseases of the anus and rectum	1070	78	0407020390	Body removed – rectum or colon polyps	145	13
C18	Malignant neoplasms of the colon	255	19	0407020063	Partial colectomy	135	12
K59	Other functional intestinal disorders	66	5	0407020209	Enteroctomy and/or resection	128	11
L52	Erythema nodosum	27	2	0407020101	Colostomy	105	9
K56	Paralytic ileus and intestinal obstruction without hernia	3	0.2	0407020128	Dilatation of the anus and/or rectum	91	8
K51	Ulcerative colitis	2	0.1	0407020098	Abdominal colporrhaphy	51	5
C63	Malignant neoplasms of other and unspecified male genital organs	1	0.1	0407020241	Enterostomy closure	50	4
				0407020187	Enteroanastomy	41	4

Enterotomy is surgical incision into the intestine. ICs: intestinal complications; ICD: International Classification of Diseases.

The age variable was calculated based on the difference between the date of birth and the first conventional therapy claim reported (index date). The age was described as continuous variable, including the mean, standard deviation, median and quartiles. For the sex and race variables, they were described as categorical variables, with absolute frequencies and percentage.

Time of follow-up was calculated based on the difference between date of first claim of conventional therapy (index date) and the last date of patient information available at database. Conventional treatment profile of UC patients was evaluated and expressed by the percentage of patient who had at least one claim of UC therapy. Patients could change, add or stopped some of the conventional therapies (sulfasalazine, mesalazine, azathioprine, methotrexate and cyclosporine) during the treatment approach, therefore, the patient may have used one or more conventional therapies during the study period. Conventional treatment initiation was expressed as the number of patients (absolute and percentage) per calendar year (2011-2020) according to the first claim of the conventional therapy in the database. Timing using conventional therapy was calculated based on the first and the last claim of the therapy presented by the patient regardless of gaps. Switch conventional therapy was considered when it had at least one claim of different conventional therapy than the previous one. The time between one conventional therapy to another conventional therapy was described as continuous variable, including the mean, standard deviation, median and quartiles of the mean.

The main outcomes were regarding ICs in patients who initiated on conventional treatment between 2011 and 2020 in the SUS. Since each individual attended different amount of time in the database, some ICs parameters were analyzed as the frequency per person-years of follow-up. For incidence rate, confidence interval was estimated by Poisson rate confidence interval. The number of claims and the number of patients with at least one claim were expressed to address the total number of claim [patient could present more than one IC (claim) for the respective group of IC] and the total number of patients with at least one IC (claim).

Missing values were reported as missing information and imputation methods were not used. When needed, patient was censored for IC analyses. For Kaplan-Meier curve the last information available of the patient or the end of the study period was considered as censored for patients who did not present IC. IC was not assessed according to the conventional treatment received and neither if patient discontinued or not discontinued the conventional therapy treatment. Data was analyzed using Python version 3.6.9.

RESULTS

In the present study, a total of 41229 patients with UC meeting the inclusion criteria were exposed to conventional medications between 2011 to 2020 in DATASUS. Most patients were female (65%) and

were reported as white (56%). The mean ± SD age was 48 ± 15.42 years old, with a mean ± SD follow-up of 3.65 ± 2.27 years. Most patients (56%) were in the Southeast region of Brazil, followed by South (21%), and Northeast (14%) (Table 2).

All patients with UC had received conventional therapy and the mean ± SD time using each was 2.64 ± 2.29 years. Regarding the conventional treatment profile for patients with UC, mesalazine had the highest proportion (87%). Azathioprine was the second (16%) and sulfasalazine the third (15%) most frequent drugs among patients with UC. Methotrexate was reported only by 1% of the patients and cyclosporine by less than 1% (Table 2).

Table 3 shows the incidence rate of bowel complications in patients with UC exposed to conventional treatment. The overall IR was 2.55 (2.46-2.63) per 100 patients. The IR of associated diseases (ICD-10-related), procedures-related and UC hospitalizations were 0.93 (0.89-0.98), 0.76 (0.71-0.80) and 0.97 (0.92-1.02), respectively. Considering that one patient could present more than one IC, a total of 6711 bowel complications claims were reported over the study period in these patients (Table 3).

Regarding the annual IC (overall and segregated types) in patients with UC, during the study period (≤ 1 year and 5 years), the overall complications rates were 2.90% at one year and 2.36% at 5 years. Segregated by type, complications related to the associated diseases (ICD-10) were1.18% and 0.88%; the procedure-related complications were 0.71% and 0.68%; and the complications related to hospitalizations due to colitis was 1.10% and 0.84%, respectively (Table 4). The mean number of events per patient was also similar over the years for both the overall and the segregated IC types in patients with UC (Table 4). A description of the time to event for overall and segregated IC type in the population with UC is shown in Figure 1. In the Kaplan-Meier analysis, the probability to present any type of ICs (overall) was approximately 20% by the end of the study period. The segregated IC types had a comparable probability to present an event (less than 10% for procedures, hospitalization and associated disease) by the end of the study period (Figure 2).

From the 1273 patients with IC due to disease associated to UC found in the study, the categories Other diseases of the anus and rectum (78%), Colon malignant neoplasm (19%), Other functional intestinal disorders (5%) were the most common ones (Table 1).

Additionally, for the 1125 patients with IC due to procedures performed in consequence of the UC progression, it was found that abdominal rectosigmoidectomy (15%), body removed-rectal or colon polyps (13%) and partial colectomy (12%) were the most common ones (Table 1).

The length of hospitalization stay was 6.62 ± 6.62 d and the number of procedures performed during a hospitalization was 0.32 ± 0.28. The most common hospitalization procedure was the treatment of non-infectious enteritis and colitis (77%), followed by diagnosis and/or emergency care at a medical clinic (7%) and treatment of other intestinal diseases (4%) (Table 5).

DISCUSSION

The present study described some specific categories of ICs in patients with UC treated with conventional therapies available in the public health system in Brazil between January 2011 and January 2020. Women, who were reported as white and from the Southeast region were the patients with the highest proportions in this study. The mean time of use of conventional therapy was 2.64 years and mesalazine was the most conventional therapy used. The mean age of UC patients treated with conventional medication in SUS was 48.86 years. This finding is similar to another database study, which reported that the highest proportion of patients with UC was in the 45-54 age group[25].

UC is considered a global burden due to its high prevalence and incidence in developed countries. Lima Martins *et al*[26], in 2014, carried out a study in Brazil, presenting a UC incidence of 5.3/100000 inhabitants/year. In São Paulo, Brazil the incidence of UC was 8/100000 inhabitants per year in 2015 [27]. A study carried out in an underdeveloped region of Northeastern in Brazil found an incidence of UC of approximately 0.6/100000 inhabitants per year in 2012[28].

Demographic characteristics of patients with UC in DATASUS showed that the large proportion of patients were from the Southeast region and the minority from the North region of Brazil. Although these regions represent one of the highest and lowest densities in the country, respectively, the number of UC cases in each region may also be due to social differences and inequalities in access to health services found in the country, in line with previous Brazilian perspectives findings reported in the literature[29].

Several studies have shown that uncontrolled UC is associated with structural damage and impaired gastrointestinal functioning[30]. This population-based study in Brazil depicted the year-by-year proportion of patients with IC under conventional therapy in SUS. During the first year and the last 5 years of follow-up, about 2.90% and 2.36%, respectively, of the patients had one or more IC in consequence of the UC progression. The rate of IC also appeared not to be high, similarly found in another international study. Thus, the proportion of patients with some complication seems to be constant over time even under treatment. It is known that continuing conventional therapy in some cases may delay more effective therapy and place patients at risk for worsening disease and complications[31].

Table 2 Demographic characteristics and follow-up of patients with ulcerative colitis treated with conventional therapies, *n* (%)

Variable	*n*	%
Patients	41229	100
Age[1], yr		
mean ± SD	48.86 ± 15.42	
Median (IQI)	48.09 (60.15-36.94)	
Sex		
Male	14417	35
Female	26812	65
Ethnicity		
White	23290	56
Black and other	11987	33
Missing	4643	11
CVT initiation		
2011	4300	10
2012	4054	10
2013	4259	10
2014	4877	12
2015	4846	12
2016	5039	12
2017	5565	13
2018	5040	12
2019	3249	8
Follow-up time[2], yr		
mean ± SD	3.65 (2.27)	
Median (IQI)	3.25 (1.75-5.26)	
Region of residence		
Southeast	22942	56
South	8831	21
Midwest	2638	6
Northeast	5833	14
North	985	2
Missing	0	0
Patients under CVT		
Mesalazine	35923	87
Sulfasalazine	6148	15
Azathioprine	6413	16
Ciclosporine	116	0
Methotrexate	290	1
Time using CVT[3], yr		
mean ± SD	2.64 (2.29)	
Median (IQI)	1.92 (0.75-4.00)	
Patients switched treatment after first	2564	6

IC[4]	
Time to switch treatment after IC, yr	
mean ± SD	0.36 (0.76)
Median (IQI)	0.08 (0.08-0.25)
Min	0.08

Values are expressed as numbers (%) or medians (range).
[1]Age at index date.
[2]Time since index date up to the last date of patient information available in the database.
[3]Calculated based on the index date and the last claim of conventional therapy.
[4]Switching from one conventional therapy to another conventional therapy.
CVT: Conventional therapy; IQI: Interquartile interval; UC: Ulcerative colitis.

Table 3 Incidence of intestinal complications (overall and segregated types) in patients with ulcerative colitis considering all population at risk

	Total of events	First event[1] (*n*)	PY[2]	IR (95%CI)
Overall	6711	3616	141997.40	2.546 (2.464-2.628)
Associated disease (ICD-10-related)	3766	1372	146806.44	0.934 (0.885-0.983)
Procedure-related	1324	1125	148326.75	0.758 (0.714-0.802)
Colitis-related Hospitalization	1791	1426	147412.62	0.967 (0.917-1.017)

Incidence rate = *n* of patients with event/patient year × 100.
[1]Counted only the first intestinal complication (claim), but the patient could present with more than one IC (represented by the sum of the first claim).
[2]Patient year = sum of time of all patients under risk, since index date up to first IC or last available data.
ICs: Intestinal complications; IR: Incidence rate; ICD: International Classification of Diseases; PY: Patient year; UC: Ulcerative colitis.

Although the rates of ICs in the present study might be underestimated due to intrinsic limitation of the method applied, the impact of the progression of UC disease with consequent complication for the health system and patient might be significant, especially due to the severity and type of the complication generated for the patient. We found that almost 4% of the UC patients performed a surgery and/or procedure of anus or rectum during the study period. Surgical complications represent a substantial burden in terms of cost and quality of life, with reoperations, medical fees and additional hospital admissions being the main cost factors[13].

The associated diseases observed in the present study were mainly represented by other diseases of rectum and anus (78%) followed by the malignant neoplasm of colon (19%). Literature shows that UC patients with severe and persistent inflammation have an increased risk of developing neoplasm of colon[32]. In the United States, the prevalence of neoplasm of colon in a patient with UC was about 3.7% [33], in Spain was 11.7%[34]. It is well known that cancer is more likely to develop in patients with extensive colitis lasting 8-10 years or more[35]. In our investigation, the average follow-up period was only 3.65 years. Thus, it is possible that our results would be improved by a longer follow-up period.

Although no direct statistical comparison was performed, the incidence rates of ICs found in the present study indicate that ICs related to diseases associated to UC (ICD-10 code) were the highest, followed by those related to the procedure and finally hospitalization for UC. Only hospitalization related to ICD-10 of UC was considered in this study, which may imply underestimated rates. That is, if the patient was hospitalized due to the progression of the disease, other ICDs-10 codes were probably used and, therefore, was not counted in this study. Even though, we might assume that conventional therapy was not able to control the inflammatory process for all patients leading them to seek urgent and emergency services, or even been hospitalized and/or require surgeries procedures.

Conventional medical treatment for UC may have limited efficacy or serious adverse reactions[36]. Currently, 20% to 40% of patients with UC do not respond to conventional therapies and may receive secondary drug treatment or even colectomy. Anti-TNF agents were the first biologics to be used in the treatment of IBD, including infliximab and adalimumab. Other biologics therapies that target specific immune pathways has been implemented in several countries, including in Brazil, as potential therapeutics for UC[37,38]. In this line, Vedolizumab was recently approved by ANVISA for the treatment of adult patients with moderate to severe UC[39] who are not responding to one or more conventional treatments, such as steroids, immunosuppressive agents or TNF blockers[36]. However, during the study period, there was no biological therapy available in the SUS.

Table 4 Annual incidence of intestinal complications (overall and segregated type) in patients with ulcerative colitis over the study period (January 2011-February 2020)

	Overall				Associated disease (ICD-10–related)				Procedure-related				UC-related hospitalization			
	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD	Patients (*n*)	%	Claims (*n*)	mean ± SD
≤ 1 yr (*n* = 41229)	1197	2.90	1582	1.32 ± 1.3	487	1.18	763	1.57 ± 1.92	293	0.71	312	1.06 ± 0.29	456	1.10	520	1.14 ± 0.44
2 yr (*n* = 36389)	931	2.56	1250	1.34 ± 1.35	355	0.98	601	1.69 ± 2.08	262	0.72	284	1.08 ± 0.36	343	0.94	376	1.1 ± 0.32
3 yr (*n* = 28795)	716	2.49	953	1.33 ± 1.39	305	1.06	493	1.62 ± 2.05	182	0.63	201	1.1 ± 0.34	242	0.84	263	1.09 ± 0.34
4 yr (*n* = 22012)	531	2.41	668	1.26 ± 1.16	219	0.99	323	1.47 ± 1.72	158	0.72	170	1.08 ± 0.37	163	0.74	177	1.09 ± 0.32
5 yr (*n* = 16510)	389	2.36	457	1.17 ± 0.88	146	0.88	190	1.3 ± 1.36	113	0.68	115	1.02 ± 0.13	139	0.84	155	1.12 ± 0.36

n of patients: Counted only the number of patients with intestinal complications (ICs) (first IC), but patients could present with more than one IC. *n* of claims: Counted the total number of claims. ICs: Intestinal complications; ICD: International Classification of Diseases; IR: Incidence rate; UC: Ulcerative colitis.

The treatments available in the SUS until 2020 involved the use of aminosalicylates (sulfasalazine and mesalazine), corticosteroids (hydrocortisone and prednisone), immunosuppressants (azathioprine, 6-mercaptopurine and (intravenous cyclosporine), and antibiotics[40]. Biological therapy was included in the national protocol of UC treatment in early 2020[40,41]. Before that period, patients who needed biological therapy had to find other strategies besides the SUS to get biologic therapies for UC treatment, such as out of pocket, private healthcare and/or even judicial action.

Limitations

The main limitations of this study are related to the retrospective design of the study and the potential information biases and attrition of this study. With regard to information bias, the lack of information can lead to underestimation of results. Longitudinal and time-dependent analyzes of data correction are more accurate, however, DATASUS data end up being limited by this. Another limitation refers to the availability of medicines by the SUS, which are not always available and/or are available at different times during the period of this study, such as corticosteroids. Use of conventional therapy was an assumption based on the first claim of each drug at the database, so the patients could have not been under treatment during all the study period. However, as a real word study, this scenario reflects the daily life of UC patients. The severity of the disease was not considered in the analyses due to methodological limitations.

Additionally, for data analysis, the inpatient database (SIH) does not have patient identification and the use of probabilistic RLK, event rates may be underestimated, as they are mainly captured in outpatient databases (SIA). As it is a descriptive observational study, it is prone to confounding factors that are difficult to control or assess. In an attempt to reduce these biases, broad terms of procedures and ICD-10s related to possible complications were used. Although the ICD-10 codes used, procedures and hospitalization were predefined as ICs, other diseases and/or procedures related to UC could be codes could have been studied as ICs proxy, however, we were not able to predict all ICD-10.

Table 5 Length of hospitalization and most common procedures performed during hospitalization

Length of hospitalization and procedures	mean ± SD	Median (IQI)	Procedures (inpatient most common procedures[1])		Patients (*n* = 1426)	%
			ICs	Description of ICs		
Hospitalization (inpatient)			303070099	Treatment of enteritis and colitis not infectious	1103	77
Length of hospital stay, d (PPPY)	6.62 ± 6.62	4.60 (2.63-8.11)	301060088	Diagnose and/or emergence care in medical clinic	102	7
Number of procedures performed (PPPY)	0.32 ± 0.28	0.23 (0.15-0.38)	303070110	Treatment of other intestinal diseases	56	4
			301060070	Diagnosis and/or emergence care in surgery clinic	47	3
			415010012	Treatment with multiple surgeries	44	3
			415020034	Other procedures with following surgeries	30	2
			407020071	Total colectomy	29	2
			303070102	Treatment of digestive tract disease	23	2
			407020330	Total proctocolectomy	12	1
			303010061	Exploratory laparotomy	9	1

[1]Inpatient procedures reported with ulcerative colitis disease International Statistical Classification of Diseases and Related Health Problems codes.
ICs: Intestinal complications; ICD: International Classification of Diseases; IQI: Interquartile interval; PPPY: Per patient per year; UC: Ulcerative colitis.

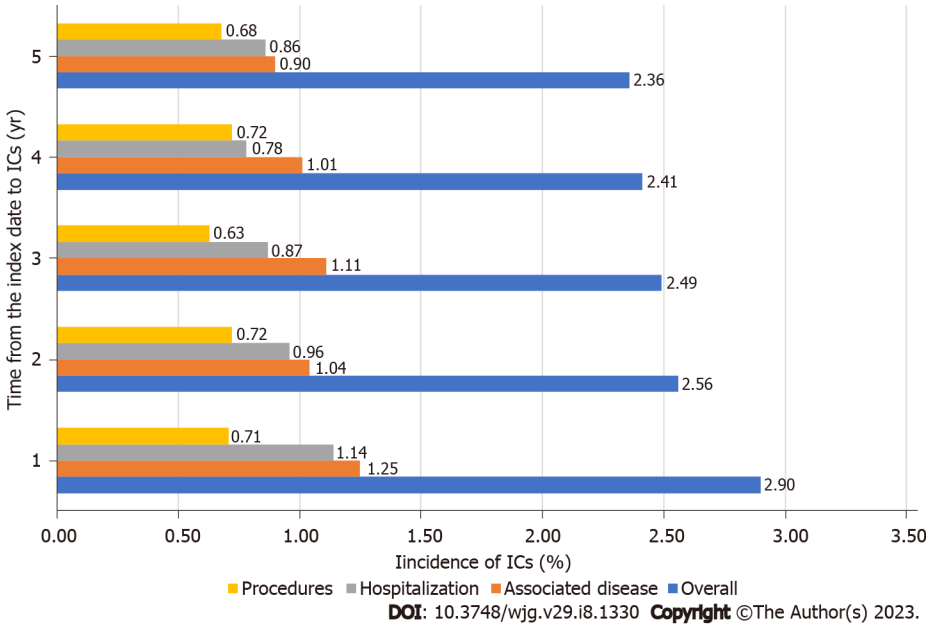

Figure 1 Annual intestinal complication (overall and segregated types) in ulcerative colitis patients over the study period (January 2011-February 2020). ICs: Intestinal complications.

CONCLUSION

UC have impact both for the population and public system (SUS) due to its clinical management and development of comorbidities and associated diseases. The results of this study highlight some issues regarding the progression of UC in patients who were treated with conventional therapies in the Brazilian public system showing that a percentage of UC patients have presented IC during the history of the disease. Most of the IC represent an important demand to the health system, with a need for health support and hospitalization. Current UC therapies, as well as innovative therapies that effectively alleviate UC symptoms, along with the implementation of an adequate program to access

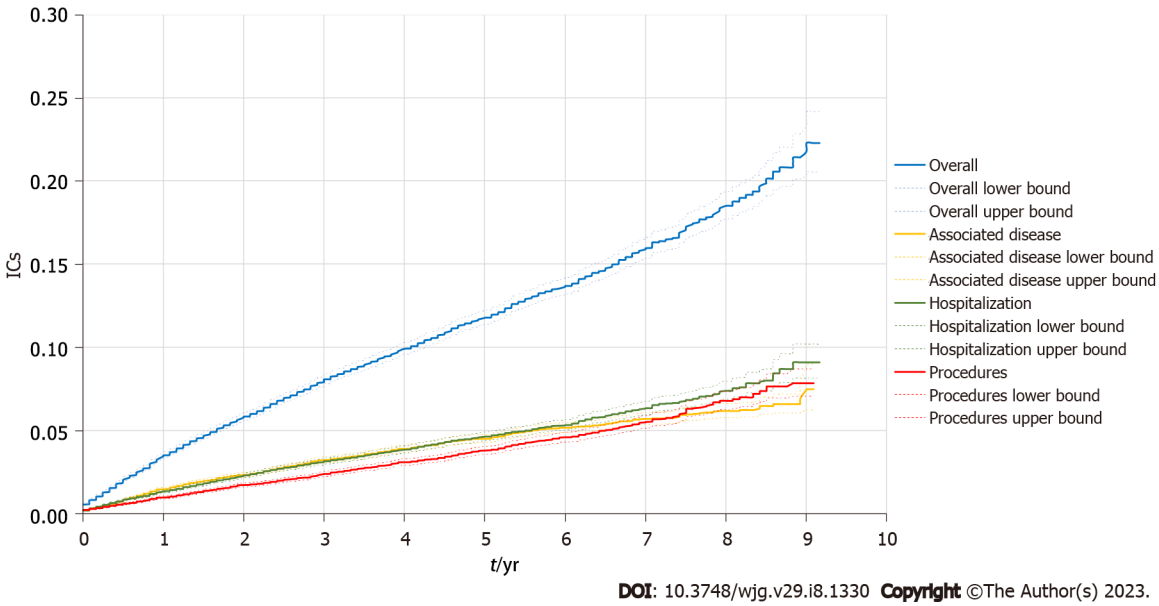

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Figure 2 Kaplan-Meier curve depicting time to intestinal complication in ulcerative colitis patients. ICs: Intestinal complications.

and manage therapy for patients with UC, may improve the control or progression of UC and consequently decrease the negative the impact of the disease.

ARTICLE HIGHLIGHTS

Research background

This was an observational, descriptive, and retrospective study from 2011 to 2020 from the Department of Informatics of the Brazilian Healthcare System database.

Research motivation

To understand the real world situation of patients with ulcerative colitis (UC) in the public healthcare system.

Research objectives

Describe the intestinal complications (IC) of patients with UC who started conventional therapies.

Research methods

Patients ≥ 18 years of age who had at least one claim related to UC International Statistical Classification of Diseases and Related Health Problems (ICD-10) code and at least 2 claims for conventional therapies were included. IC was defined as at least one claim of: UC-related hospitalization, procedures code for rectum or intestinal surgeries, and/or associated disease defined by ICD-10 codes.

Research results

In total, 41229 UC patients were included. Overall IR of IC was 3.2 cases per 100 PY. Among the IC claims, 54% were related to associated diseases, 20% to procedures and 26% to hospitalizations. The overall annual incidence of IC was 2.9%, 2.6% and 2.5% in the first, second and third year after the first claim for therapy (index date), respectively. Over the first 3 years, the annual IR of UC-related hospitalizations ranged from 0.8% to 1.1%; associated diseases from 0.9% to 1.2%.

Research conclusions

Study shows that UC patients under conventional therapy seem to present progression of disease developing some IC, which may have a negative impact on patients and the burden on the health system.

Research perspectives

Biologics were implemented in the public healthcare system after the study period, it would be interesting to verify the IC impact of this implementation in patients with UC.

FOOTNOTES

Author contributions: Martins AL, Galhardi Gasparini R, Sassaki LY, Saad-Hossne R, Barreto TB, Marcolino T and Yang Santos C participated in designed, interpretation of the data and revised the article critically for important intellectual content; Ritter AMV participated in the acquisition, analysis and draft the initial manuscript.

Institutional review board statement: In alignment with Brazilian ethical resolution number 510, 2016, ethical approval was not necessary since this is a secondary study using anonymized data.

Conflict-of-interest statement: Martins AL served on the advisory board of Takeda, AbbVie, Janssen, Pfizer and Amgen and is a speaker for Amgen and Janssen. Galhardi Gasparini R is a speaker for Janssen, Takeda and AbbVie. Sassaki LY is a speaker for Janssen and Takeda and participated in the advisory boards of Takeda and AbbVie. Saad-Hossne R is a speaker and on the advisory boards for AbbVie, Takeda, Janssen, Pfizer, Fresenius and Amgen, as well as a speaker for Novartis. AMVR is an employee of IQVIA Brazil. Marcolino T, Barreto TB and Yang Santos C are employees of Takeda Pharmaceutical Brazil.

Data sharing statement: No additional data are available.

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Country/Territory of origin: Brazil

ORCID number: Adalberta Lima Martins 0000-0002-0273-5743; Rodrigo Galhardi Gasparini 0000-0002-1032-5349; Ligia Yukie Sassaki 0000-0002-7319-8906; Rogerio Saad-Hossne 0000-0002-8166-0304; Alessandra Mileni Versut Ritter 0000-0002-4434-4791; Tania Biatti Barreto 0000-0003-1871-1815; Taciana Marcolino 0000-0003-2578-4537; Claudia Yang Santos 0000-0002-6023-607X.

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ORIGINAL ARTICLE

Observational Study

Establishment of a prediction model for severe acute radiation enteritis associated with cervical cancer radiotherapy

Chen-Ying Ma, Jing Zhao, Guang-Hui Gan, Xiao-Lan He, Xiao-Ting Xu, Song-Bing Qin, Li-Li Wang, Li Li, Ju-Ying Zhou

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Chen-Ying Ma, Jing Zhao, Guang-Hui Gan, Xiao-Lan He, Xiao-Ting Xu, Song-Bing Qin, Li-Li Wang, Li Li, Ju-Ying Zhou, Radiation Oncology, The First Affiliated Hospital of Soochow University, Suzhou 215123, Jiangsu Province, China

Ju-Ying Zhou, State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou 215123, Jiangsu Province, China

Corresponding author: Ju-Ying Zhou, MD, Radiation Oncology, The First Affiliated Hospital of Soochow University, No. 188 Shizi Street, Suzhou 215123, Jiangsu Province, China.
zhoujuyingsy@163.com

Abstract

BACKGROUND

Cervical cancer is one of the most common gynecological malignant tumors. Radiation enteritis (RE) leads to radiotherapy intolerance or termination of radiotherapy, which negatively impacts the therapeutic effect and seriously affects the quality of life of patients. If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

AIM

To analyze the relationships between severe acute RE (SARE) of cervical cancer radiotherapy and clinical factors and dose-volume parameters retrospectively.

METHODS

We included 50 cervical cancer patients who received volumetric modulated arc therapy (VMAT) from September 2017 to June 2018 in the Department of Radiotherapy at The First Affiliated Hospital Soochow University. Clinical and dose-volume histogram factors of patients were collected. Logistic regression analysis was used to evaluate the predictive value of each factor for SARE. A nomogram to predict SARE was developed (SARE scoring system ≥ 3 points) based on the multiple regression coefficients; validity was verified by an internal verification method.

RESULTS

Gastrointestinal and hematological toxicity of cervical cancer VMAT gradually increased with radiotherapy and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal swelling, and blood in the stool. There was no significant difference in the incidence of gastrointestinal toxicity between the radical and postoperative adjuvant radiotherapy groups ($P > 0.05$). There were significant differences in the small intestine V_{20}, V_{30}, V_{40}, and rectal V_{40} between adjuvant radiotherapy and radical radiotherapy after surgery ($P < 0.05$). Univariate and multivariate analyses revealed anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and disease activity index (DAI) score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) as independent predictors of SARE.

CONCLUSION

Anal bulge rating (> 0.500 grade) and DAI score (> 2.165 points) can predict SARE. The nomogram shows potential value in clinical practice.

Key Words: Cervical cancer; Intensity-modulated radiotherapy; Radiation enteritis; Nomogram; Predictor

Core Tip: Radiation enteritis (RE) not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy. The aim of our study was to determine the cumulative incidence of acute RE associated with cervical cancer radiotherapy in patients with RE in organs at risk and changes in dose-volume histogram indices. The nomogram of severe acute RE (SARE) was further developed according to the clinical factors, cumulative incidence of SARE and dosimetric parameters of volumetric modulated arc therapy patients, which may be useful for individualized risk assessment and accurate prediction of SARE to guide clinical treatment strategies.

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INTRODUCTION

Cervical cancer is one of the most common gynecological malignant tumors, ranking fourth among malignant tumors in females worldwide[1]. Radiotherapy plays an important role in the postoperative adjuvant treatment of cervical cancer patients and the radical treatment of patients who are ineligible for surgery. Radiation enteritis (RE) is a common and potentially dose-limiting toxic reaction of pelvic radiotherapy. The clinical manifestations include nausea, vomiting, abdominal pain, bloody stool, mucous stool, diarrhea, tenesmus, and urinary incontinence. RE not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy, which negatively impacts the therapeutic effect. If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

Many studies have shown that the occurrence and development of RE are related to intestinal radiotherapy technology, total dose, total volume, fraction dose, ratio of dose to volume, and uniformity of radiation dose distribution[2-6]. RE is also influenced by factors such as inflammatory bowel disease [7], collagen vascular disease[8], history of abdominal and pelvic surgery[9], history of pelvic inflammatory disease, diabetes mellitus[10], and acquired immunodeficiency syndrome[11]. However, there is no consensus on the relative importance of these predictors. Only a few studies have explored the dosimetric risk factors for RE in cervical cancer patients[12]. How to predict RE and reduce adverse reactions is the core issue of clinical radiotherapy for cervical cancer. Notably, a single predictor has limited predictive power and cases can show substantial differences in heterogeneity of RE; these issues should be addressed in further research[13]. To specifically and accurately identify RE, there is an urgent need for an accurate predictive model that combines multiple factors.

The initial objective of this study was to determine the cumulative incidence of acute RE associated with intensity modulated radiation therapy (IMRT) for cervical cancer in patients with RE in organs at risk (OAR) and changes in dose-volume histogram indices. The severe acute RE scoring system (SARE-

SS) was defined by integrating clinical factors and dosimetric physical parameters. Finally, the nomogram of severe acute RE (SARE) was further developed according to the clinical factors, cumulative incidence of SARE and dosimetric parameters of volumetric modulated arc therapy (VMAT) patients. This nomogram may be useful for individualized risk assessment and accurate prediction of SARE to guide clinical treatment strategies.

MATERIALS AND METHODS

Patient selection

Fifty patients with cervical cancer who received pelvic local radiotherapy in the Department of Radiation Oncology of The First Affiliated Hospital of Soochow University from August 2017 to August 2018 were selected. The inclusion criteria were as follows: (1) 18-80 years old; (2) cervical cancer confirmed by pathology; (3) no history of intestinal diseases or metabolic diseases; and (4) good understanding and communication skills. The exclusion criteria were as follows: (1) Severe heart, liver and kidney dysfunction; (2) termination of treatment because of serious complications during and after radiotherapy (such as cardiopulmonary, hepatic and renal insufficiency, severe infection, severe bone marrow suppression, massive hemorrhage, vaginorectal fistula and vaginovesical fistula); and (3) refusal to participate in the study.

This study was approved by the ethics committee of The First Affiliated Hospital of Soochow University Medical Ethics Committee [approval No. 2016(100)]. All enrolled patients provided signed informed consent.

External irradiation method for cervical cancer

Radiotherapy simulation positioning: On the night before radiotherapy, all patients took laxatives to clean the intestinal track. One hour before positioning, the bladder was emptied and patients drank 800 mL of water to fill the bladder. The patients then took the supine position. A Philips [Brilliance computed tomography (CT) Big Bore] large aperture CT machine was used for simulated CT localization, and enhanced CT scanning was performed with a slice thickness of 5 mm. The scanning range was from the upper edge of T11 vertebral body to 5 cm below the ischial tuberosity. The CT localization images were transferred to the treatment planning system (V5.1.1, Elekta, Monaco, Sweden).

Radiotherapy target delineation: Magnetic resonance imaging (MRI), CT or positron emission tomography-CT (PET-CT) examinations were routinely performed during the pre-radiotherapy evaluation. The target volume and OAR were delineated according to the standards of Radiation Therapy Oncology Group and Japan Clinical Oncology Group.

Radiotherapy plan design: The Monaco treatment planning system was used to design the 7-field reverse dynamic VMAT plan using unified parameters. The X-ray beam energy was 6 MV. At least 95% of the pelvic lymphatic drainage (PTV) is required to reach the prescribed dose, and no hot spot of ≥ 110% dose can occur outside the PTV. The OAR dose limit was uniform (OAR dose limitation: Bladder $V_{40} < 0\%$, rectum $V_{40} < 40\%$, colon $V_{40} < 30\%$, small intestine $V_{40} < 20\%$, femoral head $V_{45} < 5\%$).

Prescription dose of the VMAT target volume after operation: We administered 45 Gy/25 fractions for moderate risk included lymph node metastasis, paracytal invasion, and positive margin) PTV1 and 50 Gy/25 fractions for high risk PTV1 50. 4 Gy/28 fractions, the prescription dose of the remaining target volume can be executed according to the radical external irradiation scheme.

Prescription dose of the radical VMAT target volume: We administered the following doses: PTV1 45 Gy/25 fractions, parametrial area (PTV2) 50 Gy/25 fractions, and positive lymph nodes (PGTVnd) 56 Gy/28 fractions. In cases with para-aortic area, the dose for the para-aortic lymphatic drainage area (PTV3) was 36-40 Gy/20 fractions.

Implementation of the VMAT plan: VMAT was performed using the Elekta Synergy linear accelerator. All patients were treated five times a week, with one treatment a day. Cone-beam CT (Elekta) image-guided VMAT was performed at least once a week, and the error of each treatment was kept within 3 mm.

Chemotherapy for cervical cancer

Of the 50 cervical cancer patients included in this study, 18 were treated with concurrent chemoradiotherapy (CCRT) and 32 received sequential chemoradiotherapy (SCRT). All doses and adjustments of the chemotherapy regimen followed the NCCN guidelines[14].

End point definitions

General data collection: Data on age, weight, height, body mass index (BMI)[15], age-adjusted Charlson Comorbidity Index (aCCI), pathological data, HPV, squamous cell carcinoma antigen, risk rating, FIGO

2018 staging information, surgery, radiotherapy and chemotherapy were collected.

Data collection of acute RE: The adverse reactions of all patients were monitored at baseline and 2 wk, 4 wk, and 6 wk after the start of radiotherapy. Adverse reactions included diarrhea, abdominal pain, colitis, anal distension, hematochezia and bone marrow suppression. The diagnosis and classification of RE were determined following the National Institutes of Health Common Adverse Events Evaluation Criteria (CTCAE 5.0).

We established the SARE-SS to reflect the adverse reaction score as follows: SARE-SS = the sum of CTCAE scores of diarrhea + abdominal pain + colitis + anal bulging + hematochezia. A score of ≥ 3 indicated the presence of SARE. The diagnosis of SARE was determined by at least two experienced radiation oncologists on the basis of clinical symptoms and changes in the results of ancillary tests.

Disease activity index determination: The disease activity index (DAI) is based on scores that reflect the weight loss of patients (weight unchanged is 0, 1%-5% is 1 point, 5%-10% is 2 points, 10%-15% is 3 points, more than 15% is 4 points), stool viscosity (normal is 0 points, loose stool is 2 points, diarrhea is 4 points) and stool bleeding (normal is 0 points, occult blood positive is 2 points, dominant bleeding 4 points). The sum of the three scores is divided by 3 to obtain the final DAI score.

Radiotherapy planning parameter collection: Statistics of different OAR dose-volume relationship (percentage of PTV volume receiving prescription dose), recorded as V_x.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. The quantitative data of normal distribution were expressed as (mean ± SD), and *t* test was used for comparisons between groups. The data of skew distribution were tested by Mann-Whitney *U* test. Qualitative data were expressed as number of cases and percentage; Fisher's exact probability method or χ^2 test was used to compare unordered categorical data, and Mantel-Haenszel χ^2 and Mann-Whitney *U* test were used to compare ordered categorical data. $P < 0.05$ was considered to indicate statistical significance. Receiver operating characteristic curve (ROC curve) was used to analyze the specificity and sensitivity of OAR dose-volume parameters in predicting RE. Two-factor repeated-measures ANOVA was used to analyze the relationship between DAI scores and time in different groups, and one-way ANOVA was used to compare the differences of DAI scores among four time points in each group.

To establish the prediction model, univariate logistic regression model was used to evaluate the predictive ability of each factor for SARE. Multivariate analysis was performed for significant factors from the univariate analysis. Kendall correlation analysis was used to avoid multicollinearity between factors. Factors with significant predictive value in the multivariate analysis were used to construct a nomogram. The area under the ROC curve (AUC), calibration curve and decision curve analysis (DCA) were used for nomogram validation. Data analysis was performed using R software (software version 4.0.2, R package version rmda 1.6).

RESULTS

Clinical characteristics of patients

This study included 50 patients with cervical cancer. Among the 50 patients, 14 patients received radical radiotherapy, 35 patients received postoperative adjuvant pelvic radiotherapy and 1 patient received pelvic radiotherapy combined with after loading vaginal radiotherapy because the vaginal margin was 2 cm away from the tumor boundary. The specific clinical factors are detailed in Table 1. The mean age of the postoperative adjuvant radiotherapy group was significantly lower than that of radical radiotherapy group ($P < 0.05$) (Table 2). The BMI of the postoperative adjuvant radiotherapy group was also significantly lower ($P < 0.05$), but there was no significant difference in weight, pathological type, cumulative incidence of RE and SARE and irradiation of para-aortic extension field between the two groups ($P > 0.05$).

Dose-volume comparison of OAR in the VMAT plan between the postoperative adjuvant radiotherapy group and radical radiotherapy group

Patients in the radical radiotherapy group were treated with total pelvic and parauterine local radiotherapy, and patients in the postoperative adjuvant radiotherapy group were treated with total pelvic prophylactic radiotherapy. The dose and volume of small intestine in the radical radiotherapy group were significantly reduced compared with that in the postoperative adjuvant radiotherapy group, and the decrease of V_{20}, V_{30} and V_{40} were statistically significant ($P < 0.05$) (Table 3). Rectal V_{30}, V_{35}, and V_{40} in the radical radiotherapy group were significantly higher than those in the postoperative adjuvant radiotherapy group. The increase of V_{40} was statistically significant ($P < 0.05$). There was no significant difference in the dose-volume relationship of colon, bladder and femoral head between the two groups ($P > 0.05$).

Table 1 Baseline characteristics of all patients (*n* = 50)

Characteristic	*n* (%)
Age, median (IQR)	51.0 (45.5-62.0)
Weight, IQR	55.0 (50.5-61.8)
BMI/kg/m^2, IQR	21.5 (20.7-22.9)
aCCI	
2-3/4-5	38 (76)/12 (24)
Pathological diagnosis	
Squamous cell carcinoma	47 (94)
Adenocarcinoma	1 (2)
Adenosquamous carcinoma	2 (4)
T	
> 4 cm/≤ 4 cm	24 (48)/26 (52)
N	
Negative/positive	15 (30)/35 (70)
Metastatic pelvic lymph nodes	
Positive/negative	14 (28)/36 (72)
Metastatic common iliac lymph nodes	
Positive/negative	5 (10)/45 (90)
Para-aortic lymph nodes	
Positive/negative	2 (4)/48 (96)
FIGO staging	
I/II/III/IVA	19 (38)/21 (42)/5 (10)/5 (10)
LVSI	
Positive/negative	16 (32)/34 (68)
Degree of differentiation	
Low and medium differentiation/high differentiation	40 (80)/10 (20)
Degree of infiltration	
Shallow 1/3/Medium 1/3/Deep 1/3	10 (20)/16 (32)/24 (48)
Incised margin	
R1 + R2/R0	5 (10)/45 (90)
Danger degree	
Low/medium/high risk	15 (30)/12 (24)/23 (46)
HPV	
Positive/negative	46 (92)/4 (8)
SCC	
Abnormally elevated/no abnormality observed	17 (34)/33 (66)
RE	
Occurred/not occurred	42 (84)/8 (16)
SARE/Non-SARE	15 (30)/35 (70)
Operation	
Radical surgery/no surgery	36 (72)/14 (28)
Chemotherapy	

CCRT/SCRT	18 (36)/32 (64)
Pelvic External Radiation Dose, IQR	45.0 (45.0-48.0)
Para-aortic extension field	
Radiotherapy/no radiotherapy	4 (8)/46 (92)

aCCI: Adjusted Charlson Comorbidity Index; SARE: Severe acute radiation enteritis; CCRT: Concurrent chemoradiotherapy; SCRT: Sequential chemoradiotherapy; SCC: Squamous cell carcinoma; HPV: Human papillomavirus.

Table 2 Comparison of general data between postoperative adjuvant radiotherapy and radical radiotherapy for patients with cervical cancer, *n* (%)

Factor	Postoperative adjuvant radiotherapy group	Radical radiotherapy group	T/χ^2 values	*P* value
Age/yr	48.25 ± 8.05	61.86 ± 13.17	-4.450	< 0.001
Young and middle-aged	33 (92)	5 (36)	17.301	< 0.001
Old age	3 (8)	9 (64)		
Weight	57.31 ± 8.00	53.07 ± 8.74	1.638	0.108
BMI/kg/m^2	21.86 ± 2.07	20.21 ± 2.79	2.284	0.027
Tumor type				0.186[1]
Squamous cell carcinoma	35 (97)	12 (86)		
Adenocarcinoma	0 (0)	1 (7)		
Adenosquamous carcinoma	1 (3)	1 (7)		
CCRT			0.397	0.529
Implement	12 (33)	6 (43)		
Not implemented	24 (67)	8 (57)		
Para-aortic extended field radiotherapy				0.186[1]
Implement	1 (3)	2 (14)		
Not implemented	35 (97)	12 (86)		
RE			-	1.000[1]
Occurred	30 (83)	12 (86)		
Did not occur	6 (17)	2 (14)		
SARE			2.286	0.131
Occurred	13 (36)	2 (14)		
Did not occur	23 (64)	12 (86)		

[1]Fisher exact probability method.
Data are shown as *n* (%) or mean ± SD. SARE: Severe acute radiation enteritis; CCRT: Concurrent chemoradiotherapy.

Comparison of the cumulative incidence of VMAT-related acute RE in cervical cancer between the postoperative adjuvant radiotherapy group and the radical radiotherapy group

The main radiotherapy-related adverse reactions included diarrhea, abdominal pain, colitis, anal distension, hematochezia and bone marrow suppression. All adverse reactions were grade 1 to 3; no adverse reactions above grade 3 occurred. RE was monitored as follows (Table 4). There was no significant difference in the incidences of adverse reaction between the radical radiotherapy group and postoperative adjuvant radiotherapy group ($P > 0.05$).

Evaluation of the specificity and sensitivity of OAR dose-volume parameters in the IMRT plan for predicting acute/SARE

ROC curve was used to analyze the specificity and sensitivity of small intestine V_{20}, V_{30}, V_{40} and rectum

Table 3 Organs at risk dose-volume comparison between adjuvant radiotherapy (*n* = 36) and radical radiotherapy (*n* = 14) after cervical cancer surgery (%)

OAR	Group	V_{20}	V_{25}	V_{30}	V_{35}	V_{40}
Small intestine	Postoperative adjuvant radiotherapy group	77.41 ± 16.49	51.90 ± 10.99	39.51 ± 8.24	26.40 ± 5.50	19.25 ± 4.12
	Radical radiotherapy group	67.75 ± 14.10	50.11 ± 9.86	32.92 ± 7.91	25.18 ± 7.12	15.07 ± 4.65
	Z value	-2.377	-0.864	-2.701	-0.367	-3.003
	P value	0.017	0.387	0.007	0.713	0.003
Colon	Postoperative adjuvant radiotherapy group	100.00 ± 0.00	99.88 ± 0.73	98.26 ± 6.09	91.36 ± 11.67	75.64 ± 12.59
	Radical radiotherapy group	95.57 ± 16.59	95.34 ± 17.44	94.21 ± 21.66	91.09 ± 21.12	84.17 ± 7.94
	Z value	-1.604	-0.732	-0.332	-0.750	-2.247
	P value	0.109	0.464	0.740	0.453	0.025
Rectum	Postoperative adjuvant radiotherapy group	100.00 ± 0.00	100.00 ± 0.00	99.17 ± 2.90	95.63 ± 9.41	75.64 ± 12.59
	Radical radiotherapy group	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.60 ± 1.48	84.17 ± 7.94
	Z value	0.000	0.000	-1.102	-1.745	-2.247
	P value	1.000	1.000	0.270	0.081	0.025
Bladder	Postoperative adjuvant radiotherapy group	70.87 ± 28.16	68.75 ± 24.41	47.18 ± 23.63	35.58 ± 16.04	27.33 ± 12.22
	Radical radiotherapy group	74.08 ± 27.93	71.94 ± 25.87	52.54 ± 28.93	40.40 ± 21.04	31.18 ± 16.28
	T-value	-0.354	-0.523	-0.519	-0.605	-0.627
	P value	0.723	0.601	0.604	0.545	0.531
Femoral head	Postoperative adjuvant radiotherapy group	48.55 ± 28.80	34.45 ± 22.27	24.73 ± 16.00	18.12 ± 11.68	12.23 ± 8.10
	Radical radiotherapy group	39.60 ± 19.76	27.13 ± 14.03	19.54 ± 10.07	14.42 ± 7.69	9.47 ± 5.37
	T-value	-0.951	-0.929	-0.994	-0.929	-0.983
	P value	0.342	0.353	0.320	0.353	0.326

OAR: Organs at risk.

V_{40} in predicting acute/SARE. The AUC was less than 0.60 and $P > 0.05$. These results suggest that a single OAR dose-volume parameter is not enough to predict the occurrence of acute/SARE.

DAI score changes

The DAI scores of the radical radiotherapy group were higher than those of the postoperative adjuvant radiotherapy group at the second week, fourth week and the end of radiotherapy (Figure 1). There was an interaction effect between different groups and time ($\chi^2 = 77.238$, $P < 0.01$). The DAI scores of the two groups showed an increasing trend over time ($P < 0.05$), and the DAI scores of the radical radiotherapy group increased the most.

Establishment of the SARE prediction model

Univariate analysis showed that abdominal pain, colitis, anal bulging, hematochezia, DAI score, age, and CCRT were significantly correlated with the occurrence of severe acute RE (all $P < 0.05$) (Table 5). Kendall analysis showed a relatively strong correlation between abdominal pain, hematochezia and DAI score ($R = 0.715$, 0.622, $P < 0.001$); the correlation between other factors was very weak (Figure 2A). To avoid multicollinearity, abdominal pain, hematochezia, and DAI scores were included in the multivariate analysis. Multivariate logistic regression analysis was conducted to analyze the factors of colitis, anal bulge, DAI score and age. In multivariate analysis, anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and DAI score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) were independent predictors of SARE (Table 6). These factors were used to construct the nomogram. The constant in the logistic regression equation was -10.039, and the logistic regression equation was Logit (P) = 3.988 × DAI + 2.693 × anal bulge rating -10.039.

Table 4 Comparison of the incidence of acute radiation enteritis in patients with cervical cancer treated with adjuvant radiotherapy (*n* = 36) and radical radiotherapy (*n* = 14), *n* (%)

Adverse reaction	Group	Level 0	Level 1	Level 2	Level 3	χ^2 values	*P* value
Diarrhea	Postoperative adjuvant radiotherapy group	10 (28)	19 (53)	5 (14)	2 (5)	2.748	0.432
	Radical radiotherapy group	3 (21)	10 (72)	0 (0)	1 (7)		
Abdominal pain	Postoperative adjuvant radiotherapy group	21 (58)	12 (33)	3 (9)	0 (0)	1.423	0.491
	Radical radiotherapy group	8 (57)	6 (43)	0 (0)	0 (0)		
Colitis	Postoperative adjuvant radiotherapy group	25 (69)	11 (31)	0 (0)	0 (0)	-	0.140[1]
	Radical radiotherapy group	13 (93)	1 (7)	0 (0)	0 (0)		
Anal bulging	Postoperative adjuvant radiotherapy group	22 (61)	9 (25)	5 (14)	0 (0)	1.374	0.503
	Radical radiotherapy group	11 (79)	2 (14)	1 (7)	0 (0)		
Blood in the stool	Postoperative adjuvant radiotherapy group	23 (64)	13 (36)	0 (0)	0 (0)		0.179[1]
	Radical radiotherapy group	12 (86)	2 (14)	0 (0)	0 (0)		
Myelosuppression	Postoperative adjuvant radiotherapy group	12 (33)	9 (25)	10 (28)	5 (14)	3.691	0.297
	Radical radiotherapy group	3 (21)	4 (29)	2 (14)	5 (36)		

[1]Fisher exact probability method.

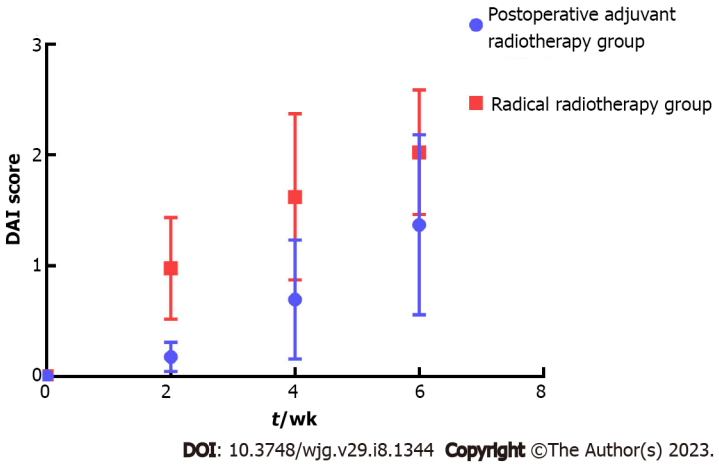

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Figure 1 Disease activity index score during volumetric modulated arc therapy in 50 patients with cervical cancer.

Development and validation of the nomogram

Based on the multivariate logistic regression coefficients, the prediction model was visually represented as a nomogram (Figure 2B). The ROC curves for the anal bulge rating, DAI score and nomogram are shown in Figure 2C. ROC analysis showed that the AUC of the prediction model was 0.950 (95%CI: 0.891-1.000), which was much higher than that of each parameter alone (anal bulge rating: 0.805, 95%CI: 0.651-0.959; DAI score: 0.892, 95%CI: 0.873-1.000). The MAL thresholds for anal bulge rating and DAI score were 0.5 and 2.165 points. The nomogram had a high predictive efficiency (sensitivity: 80.0%, specificity: 91.4%). In addition, the calibration curve showed good agreement between the predicted severe acute RE and the actual observations (Figure 2D). In most threshold probabilities, DCA showed a positive net benefit with a satisfactory nomogram, indicating a good potential clinical effect of the model (Figure 2E).

Table 5 Comparison of general data between patients with cervical cancer with severe acute radiation enteritis or no severe acute radiation enteritis

Factor	SARE (*n* = X)	No SARE (*n* = X)	OR (95%CI)	*P* value
	Median (IQR)	Median (IQR)		
Clinical factors				
Elderly (> 60 yr)/young and middle-aged (20-60 yr)			0.123 (0.015-1.040)	0.054
Age	47.5 (37.0-54.0)	53.0 (46.0-63.0)	0.930 (0.872-0.992)	0.027
Weight	53.0 (51.0-60.9)	56.0 (50.0-61.8)	0.983 (0.918-1.054)	0.631
BMI/kg/m^2	20.5 (19.7-22.2)	21.9 (20.4-23.0)	0.936 (0.746-1.174)	0.566
ACCI (2-3/4-5)			0.123 (0.015-1.040)	0.054
Tumor type (squamous/non-squamous)			0.848 (0.139-5.179)	0.859
T (> 4 cm/≤ 4 cm)			0.510 (0.153-1.695)	0.272
N (positive/negative)			0.615 (0.165-2.298)	0.538
Metastatic pelvic lymph nodes (positive/negative)			0.694 (0.185-2.611)	0.589
Common iliac lymph node metastasis (positive/negative)			0.427 (0.046-3.980)	0.455
Para-aortic lymph nodes (positive/negative)			1.471 (1.216-1.779)	0.999
FIGO stage (IIB-IVA/IB-IIA)			1.108 (0.328-3.739)	0.869
LVSI (positive/negative)			0.548 (0.147-2.034)	0.368
Differentiation degree (low and medium differentiation/high differentiation)			1.011 (0.225-4.536)	0.988
Infiltration degree (shallow 1/3/medium depth 1/3)			0.837 (0.191-3.673)	0.813
Incisal margin (R1 + R2/R0)			0.427 (0.046-3.980)	0.455
Risk (low risk/medium high risk)			1.227 (0.340-4.242)	0.754
HPV (positive/negative)			1.324 (0.127-13.785)	0.815
SCC (abnormally elevated/not abnormal)			0.303 (0.074-1.245)	0.098
Surgery (performed/not performed)			2.080 (0.497-8.706)	0.316
CCRT (implemented/not implemented)			6.042 (1.681-21.718)	0.006
Myelosuppression (Grade 1-3/Grade 0)			0.617 (0.178-2.144)	0.446
Diarrhea (Grade 1-3/Grade 0)			NA	0.999
Abdominal pain (Grade 1-3/Grade 0)			25.375 (4.750-135.559)	< 0.001
Colitis (Grade 1-3/Grade 0)			38.500 (6.530-226.993)	< 0.001
Anal bulge (Grade 1-3/Grade 0)			5.185 (1.470-18.286)	0.010
Hematochezia (Grade 1-3/Grade 0)			NA	< 0.001
DAI score	2.8 (2.3-3.0)	1.7 (1.2-2.0)	152.546 (6.045-3849.771)	0.002
Physical dose parameters				
Small intestine				
V_{20} (%)	80.9 (68.6-93.7)	78.0 (66.8-82.5)	1.015 (0.975-1.056)	0.475
V_{25} (%)	58.2 (46.0-62.9)	52.0 (48.0-57.3)	1.014 (0.955-1.076)	0.659
V_{30} (%)	41.0 (35.1-47.6)	39.6 (31.1-42.1)	1.039 (0.961-1.122)	0.335
V_{35} (%)	29.6 (23.5-32.2)	26.9 (24.4-29.1)	1.032 (0.927-1.149)	0.569
V_{40} (%)	19.5 (16.8-23.3)	19.3 (13.3-20.7)	1.068 (0.928-1.229)	0.358
Rectum				
V_{20} (%)	80.9 (68.6-93.7)	78.0 (66.8-82.5)	1.015 (0.975-1.056)	0.475

V_{25} (%)	58.2 (46.0-62.9)	52.0 (48.0-57.3)	1.014 (0.955-1.076)	0.659
V_{30} (%)	41.0 (35.1-47.6)	39.6 (31.1-42.1)	1.039 (0.961-1.122)	0.335
V_{35} (%)	29.6 (23.5-32.2)	26.9 (24.4-29.1)	1.032 (0.927-1.149)	0.569
V_{40} (%)	19.5 (16.8-23.3)	19.3 (13.3-20.7)	1.068 (0.928-1.229)	0.358
Colon				
V_{20} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	NA	NA
V_{25} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	NA	NA
V_{30} (%)	100.0 (100.0-100.0)	100.0 (100.0-100.0)	0.982 (0.775-1.246)	0.883
V_{35} (%)	100.0 (99.1-100.0)	100.0 (100.0-100.0)	0.981 (0.915-1.052)	0.981
V_{40} (%)	80.4 (71.3-89.2)	79.3 (72.1-87.8)	1.005 (0.956-1.056)	0.851
Femoral head				
V_{20} (%)	35.4 (19.7-57.3)	48.6 (22.9-65.2)	0.994 (0.971-1.017)	0.586
V_{25} (%)	24.8 (13.7-40.1)	31.6 (16.0-45.1)	0.996 (0.967-1.026)	0.797
V_{30} (%)	17.7 (9.8-29.6)	22.6 (11.4-32.6)	0.997 (0.957-1.039)	0.877
V_{35} (%)	13.3 (7.4-20.0)	16.9 (8.6-24.5)	0.989 (0.935-1.047)	0.711
V_{40} (%)	8.9 (4.9-14.3)	11.3 (5.7-16.3)	0.993 (0.916-1.076)	0.858
Total pelvic lymphatic drainage dose (Gy)	45.0 (45.0-45.0)	45.0 (45.0-48.5)	1.194 (0.877-1.625)	0.261
Para-aortic extended field radiotherapy (performed/not performed)			1.485 (1.222-1.804)	0.999

ACCI: Adjusted Charlson Comorbidity Index; CCRT: Concurrent chemoradiotherapy; SCC: Squamous cell carcinoma; HPV: Human papillomavirus; NA: Not available; DAI: Disease activity index.

Table 6 Multivariate analysis of influencing factors of severe radiation enteritis in 50 patients with cervical cancer treated with radiotherapy

	B	SE	Exp (B)	95%CI		Wald χ^2	*P* value
				Lower	Upper		
DAI score	4.106	1.371	60.705	4.134	891.506	9.463	0.003
Anal bulge rating	2.925	1.229	18.636	1.677	207.114	7.664	0.017

DAI: Disease activity index; 95%CI: 95% confidence interval.

DISCUSSION

Nearly 80% of patients receiving pelvic radiotherapy for cervical cancer have early acute toxicity and 20% have late toxicity[16]. Acute RE often occurs in the second week after the start of radiotherapy and reaches a peak in the fourth to fifth week. The clinical manifestations are nausea, vomiting, abdominal pain, bloody stool, mucous stool, diarrhea, tenesmus, and incontinence. In gynecological tumor radiotherapy, the clinical application of IMRT shows an increasing trend. This radiotherapy technology can not only obtain satisfactory target coverage dose, but also effectively reduces the radiation dose of OAR[17,18], such as V45 of rectum and small intestine[19,20], and reduces the toxic reaction of bone marrow[21,22]. Although the incidence and severity of RE have decreased under the current radiotherapy technology, these issues cannot be ignored. In addition, mild RE is often underestimated or ignored due to the lack of clinical records and inadequate assessment of toxicity[23].

In this study, we preliminarily demonstrated the close relationship between the anal bulge rating and the DAI score and SARE. More importantly, this study is the first to develop and validate an easy-to-understand visual nomogram prediction model for cervical cancer radiotherapy patients and doctors, providing more personalized and accurate SARE prediction for cervical cancer patients undergoing radiotherapy. Many studies have reported that the most important factor affecting radiation proctitis is the total dose received by the rectum, and it is also affected by the radiotherapy technique, fraction dose, total dose received by the rectum/total volume, dose volume ratio of rectum to radiation dose,

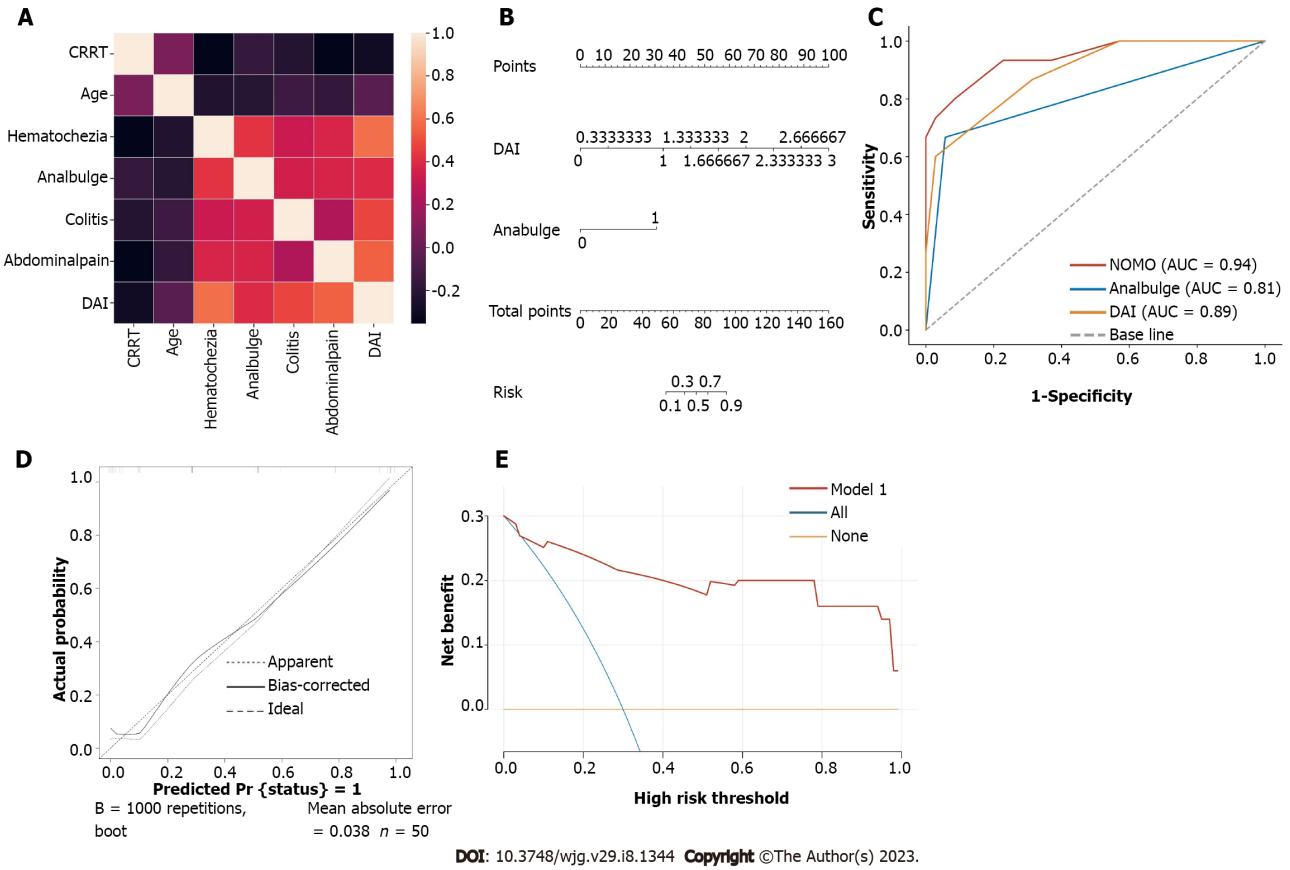

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Figure 2 Development and validation of the nomogram. A: Kendall's rank correlation analyses among factors with $P < 0.05$ in univariate logistic regression; B: Nomogram predicting the occurrence of severe acute radiation enteritis (SARE). For each individual patient, two lines are drawn upward to determine the points received from the two variables in the nomogram; the sum of these points is located on the "Total Points" line, and a line is drawn downward to determine the likelihood of this patient to have SARE; C: Receiver operating characteristic curves of anal bulge rating. Disease activity index score *vs* the predictive model; D: Calibration curves of the nomogram predicting the occurrence of SARE. The x-axis and y-axis indicate the predicted and actual probabilities of having SARE, respectively; E: Decision curves of the nomogram predicting the occurrence of SARE. The x-axis shows the threshold probabilities. The y-axis measures the net benefit, which was calculated by adding the true positives and subtracting the false positives. NOMO: Nodal modulator; AUC: Area under the receiver operating characteristic curve; DAI: Disease activity index.

and uniformity of radiation dose distribution[4-6]. According to Seppenwoolde *et al*[24], rectal V40 ≥ 80% is a physical dose factor predicting VMAT-related rectal incontinence and diarrhea in patients with locally advanced cervical cancer. Ballari *et al*[25] suggested that small bowel V45 has predictive value for acute RE. In addition, individual factors such as IBD, collagen vascular disease, history of abdominal and pelvic surgery, history of pelvic inflammatory disease, diabetes mellitus, and acquired immunodeficiency syndrome affect the determination of radiation dose and volume[7-11]. In patients with cervical cancer, CCRT exerts radiosensitization and increases gastrointestinal toxicity by a factor of 3[18]. However, there is currently no consensus on the relative importance of these relevant predictors. In our study, the dose-volume of the small intestine receiving 20-40 Gy in the radical radiotherapy group of cervical cancer was significantly reduced compared with that in the postoperative adjuvant radiotherapy group, and the reduction of V_{20}, V_{30}, and V_{40} was statistically significant ($P < 0.05$), which was considered to be related to the anatomical displacement of organs after hysterectomy. After the removal of the tumor bed and uterus, part of the intestinal loop falls into the pelvic cavity[9], resulting in increased secondary toxicity of radiotherapy. In addition, the rectal V_{30}, V_{35}, and V_{40} in the radical radiotherapy group were significantly higher than those in the postoperative adjuvant radiotherapy group, and the increase of V_{40} was statistically significant ($P < 0.05$). The cervical cancer patients treated with radical radiotherapy in this study were FIGO IIB-IVA. CT or MRI showed obvious parametrial infiltration, and mesorectal invasion occurred in some patients, resulting in a relative increase in the volume of PTV to the left, right and dorsal side. Although we found dose-volume differences in the small intestine and rectum between groups, we were unable to demonstrate that a single physical parameter independently predicted RE or SARE. Therefore, more prospective, well-designed randomized controlled trials with larger sample sizes are needed for validation.

Acute RE is self-limited, and not all acute RE will be classified as chronic RE. The prevalence of chronic RE after 10 years of radiotherapy is 10%-20%[26]. In terms of pathogenesis, inflammation and cell death occur in the intestinal mucosa during the acute phase of RE, and sustained cytokine activation

in the submucosa leads to progressive ischemia, fibrosis, and stem cell loss[27]. In contrast, chronic RE is associated with progressive endarteritis obliterans, secondary to capillary fibrosis, leading to chronic ischemia and fibrosis of the affected bowel segments[28]. The outcome of chronic RE often induces intestinal obstruction, which is similar to the Koenig syndrome observed in Crohn's disease and is considered to be related to ileal stenosis. In view of the similarities between RE and IBD, we innovatively adopted the IBD evaluation index-DAI score. Univariate and multivariate logistic regression analysis confirmed that DAI score was independent of other physical parameters and clinical factors and could be used as a new predictor of SARE. In univariate analysis, abdominal pain, colitis, anal distension, hematochezia, DAI score, age, and CCRT were statistically correlated with the occurrence of severe acute RE ($P < 0.05$). Kendall analysis showed a relatively strong correlation between abdominal pain, hematochezia, and DAI scores. Therefore, DAI score is a convenient, non-invasive and highly beneficial diagnostic system for SARE.

The clinical manifestations of SARE are complex, and it is difficult for doctors, even with extensive clinical experience, to predict and diagnose the disease early, comprehensively and accurately. If cervical cancer can be identified and treated early before, during or after radiotherapy, the toxic and side effects of pelvic radiotherapy may be improved, and the probability of radiotherapy intolerance or even termination of radiotherapy may be reduced. The multi-factor prediction model is helpful to further improve the prediction of RE. Several prediction models have been described in previous studies, but they are still not used in clinic due to the low recognition ability of RE and the neglect of heterogeneity among different patients[29,30]. In this study, we established a SARE-SS evaluation system and found that anal bulge rating and DAI score were independent predictors of SARE through multivariate statistical analysis. The above predictors were further integrated into the nomogram to allow precise individualized SARE risk assessment for each patient. The AUC of the predictive model was 0.950, which was much higher than the two independent factors of DAI score and anal bulge score. The MAL thresholds for anal bulge rating and DAI score were 0.5 grade and 2.165 points, respectively, indicating an advantage over previous models in predicting RE. We adopted the Bootstrap validation method (including drawing the calibration curve and DCA curve), which showed a statistical advantage in a relatively small sample size statistic[31,32]. In later studies, we will expand the sample size, carry out prospective cohort studies, and use external validation methods to reduce data selection bias and increase test power.

Although our study has many advantages, there are still several limitations to be addressed. First, our sample size is small, so a large cohort is needed to further develop and validate the nomogram for predicting RE. Second, since this was a retrospective study, our study may have had a selection bias. Third, we only integrated the most important and commonly used parameters in clinic. From the perspective of precision medicine, we need to combine biological factors in the future including individual genomics, proteomics, metabolomics, microbiomics, real-time dosimetry and a wider range of clinical parameters to establish a comprehensive prediction model to improve the accuracy of RE prediction. Therefore, we will add biological factors to the analysis of related studies in later studies and need to establish a more comprehensive and specific prediction model of RE.

CONCLUSION

Our findings showed that during VMAT for cervical cancer, gastrointestinal and hematological system toxicity gradually increased with radiotherapy treatment and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal bulging and hematochezia. There were significant differences in V_{20}, V_{30}, V_{40} of small intestine and V_{40} of rectum between postoperative adjuvant radiotherapy and radical radiotherapy, which may be related to intestinal anatomical displacement caused by surgery and primary disease. However, there were no significant differences in the cumulative incidence of adverse reactions between the two groups. Anal bulge rating (> 0.500) and DAI score (> 2.165) were identified as independent predictors of SARE by multivariate analysis. The validity of our established nomogram was verified by the internal verification method. Although further external validation is needed, our results indicate that the nomogram has clinical value for the prediction and evaluation of SARE.

ARTICLE HIGHLIGHTS

Research background

Radiation enteritis (RE) not only seriously affects the quality of life of patients, but it also leads to radiotherapy intolerance or termination of radiotherapy. However, data on the clinical efficacy and side effects of volumetric modulated arc therapy (VMAT) for cervical cancer are limited.

Research motivation

If the incidence of RE in patients can be predicted in advance, and targeted clinical preventive treatment can be carried out, the side effects of radiotherapy in cervical cancer patients can be significantly reduced. Furthermore, accurate prediction of RE is essential for the selection of individualized radiation dose and the optimization of the radiotherapy plan.

Research objectives

To analyze the relationships between severe acute RE (SARE) of cervical cancer radiotherapy and clinical factors and dose-volume parameters retrospectively.

Research methods

We included 50 cervical cancer patients who received VMAT from September 2017 to June 2018 in the Department of Radiotherapy at The First Affiliated Hospital Soochow University. Clinical and dose-volume histogram factors of patients were collected. Logistic regression analysis was used to evaluate the predictive value of each factor for SARE. A nomogram to predict SARE was developed (SARE scoring system ≥ 3 points) based on the multiple regression coefficients; validity was verified by an internal verification method.

Research results

Gastrointestinal and hematological toxicity of cervical cancer VMAT gradually increased with radiotherapy and reached the peak at the end of radiotherapy. The main adverse reactions were diarrhea, abdominal pain, colitis, anal swelling, and blood in the stool. There was no significant difference in the incidence of gastrointestinal toxicity between the radical and postoperative adjuvant radiotherapy groups ($P > 0.05$). There were significant differences in the small intestine V20, V30, V40, and rectal V40 between adjuvant radiotherapy and radical radiotherapy after surgery ($P < 0.05$). Univariate and multivariate analysis revealed anal bulge rating (OR: 14.779, 95%CI: 1.281-170.547, $P = 0.031$) and disease activity index (DAI) score (OR: 53.928, 95%CI: 3.822-760.948, $P = 0.003$) as independent predictors of SARE.

Research conclusions

Anal bulge rating (> 0.500 grade) and DAI score (> 2.165 points) can predict SARE. The nomogram shows potential value in clinical practice.

Research perspectives

From the perspective of precision medicine, it will also be necessary to combine biological factors, such as individual genomics, proteomics, metabolomics, microbiomics, real-time dosimetry, and a wider range of clinical parameters to establish comprehensive predictive models. This study is a prospective study with a small sample size. In the later stage, we will expand the sample size, conduct prospective cohort studies, and use external validation methods to reduce data selection bias and increase test efficiency.

FOOTNOTES

Author contributions: Ma CY and Zhao J contributed to conceptualization and methodology; He XL and Gan GH contributed to data curation; Ma CY contributed to formal analysis; Ma CY, Zhou JY contributed to funding acquisition; Zhou JY, Xu XT, Qin SB contributed to project administration; Zhou JY, Xu XT, Qin SB, Wang LL, and Li L contributed to resources; Xu XT and Qin SB contributed to supervision; Ma CY contributed to validation and visualization; Ma CY and Zhao J contributed to roles/writing-original draft; Ma CY contributed to writing-review and editing.

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Country/Territory of origin: China

ORCID number: Ju-Ying Zhou 0000-0002-2796-6388.

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ORIGINAL ARTICLE

Observational Study

Better performance of PIVKA-II for detecting hepatocellular carcinoma in patients with chronic liver disease with normal total bilirubin

Xiang-Jun Qian, Zhu-Mei Wen, Xiao-Ming Huang, Hui-Juan Feng, Shan-Shan Lin, Yan-Na Liu, Sheng-Cong Li, Yu Zhang, Wen-Guang Peng, Jia-Rui Yang, Zhe-Yu Zheng, Lei Zhang, Da-Wei Zhang, Feng-Min Lu, Li-Juan Liu, Wei-Dong Pan

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Xiang-Jun Qian, Xiao-Ming Huang, Wen-Guang Peng, Jia-Rui Yang, Zhe-Yu Zheng, Lei Zhang, Da-Wei Zhang, Wei-Dong Pan, Department of Pancreatic Hepatobiliary Surgery, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou 510655, Guangdong Province, China

Xiang-Jun Qian, Yan-Na Liu, Feng-Min Lu, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China

Zhu-Mei Wen, Hui-Juan Feng, Sheng-Cong Li, Yu Zhang, Li-Juan Liu, Department of Laboratory Medicine, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou 350025, Fujian Province, China

Shan-Shan Lin, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, United States

Corresponding author: Wei-Dong Pan, MD, Doctor, Professor, Surgeon, Department of Pancreatic Hepatobiliary Surgery, The Sixth Affiliated Hospital of Sun Yat-sen University, No. 26 Erheng Road, Tianhe District, Guangzhou 510655, Guangdong Province, China.
panwd@mail.sysu.edu.cn

Abstract

BACKGROUND

Serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) is a promising biomarker for hepatocellular carcinoma (HCC) surveillance.

AIM

To identify the contributing factors related to the abnormal elevation of PIVKA-II level and assess their potential influence on the performance of PIVKA-II in detecting HCC.

METHODS

This study retrospectively enrolled in 784 chronic liver disease (CLD) patients and 267 HCC patients in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019. Logistic regression and the area

under the receiver operating characteristic curve (AUC) were used to evaluate the influencing factors and diagnostic performance of PIVKA-II for HCC, respectively.

RESULTS

Elevated PIVKA-II levels were independently positively associated with alcohol-related liver disease, serum alkaline phosphatase (ALP), and total bilirubin (TBIL) for CLD patients and aspartate aminotransferase (AST) and tumor size for HCC patients (all $P < 0.05$). Serum PIVKA-II were significantly lower in patients with viral etiology, ALP ≤ 1 × upper limit of normal (ULN), TBIL ≤ 1 × ULN, and AST ≤ 1 × ULN than in those with nonviral disease and abnormal ALP, TBIL, or AST (all $P < 0.05$), but the differences disappeared in patients with early-stage HCC. For patients with TBIL ≤ 1 × ULN, the AUC of PIVKA-II was significantly higher compared to that in patients with TBIL > 1 × ULN (0.817 *vs* 0.669, $P = 0.015$), while the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN was not statistically significant (0.783 *vs* 0.729, $P = 0.398$). These trends were then more prominently perceived in subgroups of patients with viral etiology and HBV alone.

CONCLUSION

Serum PIVKA-II has better performance in detecting HCC at an early stage for CLD patients with normal serum TBIL.

Key Words: Protein induced by vitamin K absence or antagonist-II; Chronic liver disease; Total bilirubin; Hepatocellular carcinoma; Diagnosis; Hepatitis B virus

Core Tip: This study demonstrated that elevated serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) were positively associated with serum total bilirubin (TBIL) in patients with chronic liver disease (CLD), and the levels of PIVKA-II in CLD patients with normal serum TBIL were significantly lower than those in CLD patients with abnormal serum TBIL. Serum PIVKA-II has better performance in detecting hepatocellular carcinoma (HCC) at an early stage for CLD patients with normal serum TBIL, which was more prominently perceived in patients with viral etiology and hepatitis B virus alone. These findings may be important for surveillance counseling of early-stage HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC), a serious issue for global health, is still the sixth most prevalently diagnosed cancer and the third highest cause of cancer death worldwide[1,2]. Chronic liver disease (CLD) induced by viral and nonviral factors has been identified as the main cause of HCC, including chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol-related liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), autoimmune liver disease (AILD), and so on, with the major risk factors varying from region to region[1-3].

In recent years, although great progress has been achieved on cancer treatment with the development by surgical resection, molecular targeted therapy, and immunotherapy[2,4-6], 5-year survival rate of HCC remains not satisfactory, with only 15%-17% as reported[7,8]. The main cause of this extremely poor prognosis is that more than half of HCC patients present with advanced stages once at diagnosis[7,8]. Detecting HCC at an early stage is an efficacious way for the improvement of long term survival of HCC patients[2,9,10].

Serum HCC biomarkers as surveillance tools remain controversial because the diagnostic accuracy of the traditional biomarker for HCC, serum alpha-fetoprotein (AFP), is unsatisfactory[8,11,12]. Serum AFP is frequently influenced by many noncancerous factors and is falsely raised in non-HCC patients who have active chronic viral and advanced liver disease[13-15]. Hence, protein induced by vitamin K absence or antagonist-II (PIVKA-II), has gained increasing attention in recent years since it was first

reported for the diagnosis of HCC in 1984[2,8,9,16]. PIVKA-II is poorly related to AFP, and the diagnostic performance of HCC by combining the two biomarkers was higher compared to AFP alone[8, 17]. In addition, the guidelines of the Japan Society of Hepatology has recommended serum PIVKA-II as one of the HCC surveillance markers in at-risk populations[18,19].

PIVKA-II could also be employed to monitor HCC prognosis, treatment response, and recurrence because of its possible associations with the tumor size, HCC progression and recurrence[20,21]. Serum PIVKA-II levels have also been reported to be influenced by several noncancerous factors, such as vitamin K deficiency[22], ALD[23-26], acute hepatic failure[27], and the use of warfarin or antibiotics[26, 28] by leading to serum PIVKA-II elevation in non-HCC patients.

However, it remains unclear what the factors are regarding different liver disease etiologies, the liver injury severity, and disease activity degree associated with abnormal PIVKA-II levels in patients with CLD and HCC. Therefore, we aimed to identify the potential contributing factors and evaluate their influence on serum PIVKA-II levels and the performance of PIVKA-II for the diagnosis of HCC in different populations.

MATERIALS AND METHODS

Patients selection

This study eventually retrospectively registered 1051 patients after excluding 80 patients from 1131 registered patients. The mean age of the total cohort was 48.93 years ± 13.70 years, and 73.6% of them were male (n = 773). Supplementary Figure 1 shows the selection flowchart and the analysis process. All patients were recruited in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019.

The patients included following the criteria as: (1) With CLD or HCC of clear etiology diagnosed by clinical or histological evidence, including chronic hepatitis B (CHB), chronic hepatitis C (CHC), ALD, NAFLD, and AILD; (2) CHB and CHC were defined as patients with positive hepatitis B surface antigen (HBsAg) and positive hepatitis C virus (HCV) antibodies and/or HCV RNA for at least 6 months; and (3) complete and detailed laboratory and clinical information, including serum PIVKA-II, relevant laboratory tests, and other clearly clinical characteristics and records. The patients excluded following the criteria as: (1) Patients diagnosed with CHB and ALD simultaneously; (2) patients with liver failure, esophageal and gastric variceal bleeding, or drug-induced liver disease; (3) patients without sufficient information on relevant laboratory tests and clinical records; (4) patients without a certain clinical diagnosis of HCC; (5) patients accepting warfarin or long-term treatment with antibiotics at the time of enrollment; and (6) patients with other malignant tumors or a prior history of antitumor treatment in HCC.

According to the Milan criteria, early-stage HCC is defined as a nodule with the diameter less than 5 cm or two to three nodules with the diameter each less than 3 cm and do not have significant vascular invasion or extrahepatic metastases[2,15,29], and beyond the Milan criteria was considered as late-stage HCC. A positive lesion discovered by the suggested imaging techniques and contrast agents or histopathological confirmation were used to determine the diagnosis of HCC[2,3,9,15,30]. For CLD patients at enrollment, the lack of an HCC was determined through that there were no any suspicious hepatic masses in clinical and imaging evidence, and if with an abnormal PIVKA-II level, a continuous imaging surveillance within the subsequent months was appropriate.

The existence of cirrhosis was generally determined *via* imaging, laboratory and clinical characteristics, or liver biopsy without a routine examination.

The ethics committee of Peking University Health Science Center granted this study approval (IRB00001052-19081). All procedures carried out in this study had followed the Helsinki declaration or equivalent ethical principles.

Study variables

Serum PIVKA-II level was quantitatively detected through the chemiluminescence enzyme immunoassay (LUMIPULSE® G1200, FUJIREBIO INC, Japan) by using Lumipulse® G PIVKA-II reaction cartridges in the clinical laboratory of Mengchao Hepatobiliary Hospital of Fujian Medical University, according to manufacturer's instructions. The lower limit of detection is > 0 mAU/mL, and the upper is 75000 mAU/mL. The upper limit of normal (ULN) was 40 mAU/mL[9]. Furthermore, abnormal elevation of serum PIVKA-II was defined as its values elevated above 40 mAU/mL (ULN).

Liver-related laboratory tests, including liver enzyme and function and routine blood, were performed in the clinical laboratory using commercially available kits by manufacturers' instructions. Test values would be reviewed if judged as unusual in order to ensure their accuracy.

Statistical analysis

SPSS 24.0 software (New York, NK, United States) was applied to finish the basic statistical analyses. The mean ± SD or median and interquartile range (IQR), as the continuous variables, were analyzed by t or Mann-Whitney test between two groups or Kruskal-Wallis test among three or more groups; χ^2 test

for categorical variables. Univariate and multivariate (forward) analyses of logistic regression were constructed to investigate factors associated with the abnormal elevation of serum PIVKA-II for non-HCC and HCC patients, respectively. Then, GraphPad Prism 7 (California, CA, United States) was used to plot the distributions of the levels of PIVKA-II in different subgroups and further compare them. The area under receiver operating characteristic (ROC) curve (AUC) and 95% confidence interval (CI), which reflecting the PIVKA-II's performance for HCC, were conducted and calculated by MedCalc version 18.2.1 (Ostend, Belgium). The best cutoff value, sensitivity, specificity, positive likelihood ratio (LR+), and negative likelihood ratio (LR-) of PIVKA-II levels were also calculated and shown. $P < 0.05$ was deemed statistically significant, and all tests and power analyses of significance were two-tailed.

RESULTS

Patient characteristics

The comprehensive clinical and laboratory characteristics of the study population are listed in Table 1. A total of 595 viral liver disease patients, 189 nonviral liver disease patients, 127 early-stage HCC patients, and 140 late-stage HCC patients were enrolled in. Compared to viral liver disease patients, patients with nonviral liver disease had significantly older mean age and higher median serum platelet (PLT) and GGT levels ($P < 0.05$); however, their male proportion and median serum ALT, AST and TBIL levels were significantly lower ($P < 0.05$). Interestingly, the abnormally elevated proportion of PIVKA-II > 40 mAU/mL (ULN) in patients with nonviral liver disease was significantly higher than that in patients with viral liver disease (20.6% *vs* 13.9%, $P < 0.050$), as well as the substantially higher median levels of serum PIVKA-II (27.0 *vs* 23.0, $P < 0.001$).

The median serum ALT, AST, ALP, GGT, and PLT levels in patients with early-stage HCC were significantly lower compared to those in patients with late-stage HCC ($P < 0.01$); and the significantly smaller tumor size and proportion of the number of tumors ≥ 2 were also observed in early-stage HCC patients (both $P < 0.001$), but they had a higher median level of serum ALB ($P < 0.05$). Furthermore, patients with early-stage HCC had a significantly lower abnormal proportion of PIVKA-II > 40 mAU/mL (ULN) than patients with late-stage HCC (60.6% *vs* 93.6%, $P < 0.001$), as well as significantly lower median serum PIVKA-II level (58 *vs* 5124, $P < 0.001$).

Identify the independent factors related to the abnormal elevation of PIVKA-II level (> 1 × ULN) in non-HCC and HCC patients

Then, we further analyzed and identified the independent factors related to the abnormal elevation of PIVKA-II level in non-HCC and HCC patients through logistic regression analyses, respectively. Table 2 and Figure 1A summarize the results of non-HCC patients. ALD etiology (OR: 9.883, 95%CI: 2.216-44.086, $P < 0.001$) was an independent positive factor associated with the abnormal elevation of serum PIVKA-II in non-HCC patients, as well as the factors of ALP/ULN (OR: 2.146, 95%CI: 1.429-3.221, $P < 0.001$) and TBIL/ULN (OR: 1.162, 95%CI: 1.080-1.250, $P < 0.001$). Table 3 and Figure 1B summarize the results of HCC patients. AST/ULN (OR: 1.759, 95%CI: 1.072-2.887, $P = 0.025$) was an independent positive factor associated with the abnormal elevation of serum PIVKA-II in HCC patients, as well as the factor of tumor size (OR: 1.349, 95%CI: 1.175-1.549, $P < 0.001$). These findings implied that there were obvious differences in the factors associated with the abnormal elevation of PIVKA-II level between non-HCC and HCC patients.

Serum PIVKA-II levels were different among different subgroups of patients with non-HCC, early-stage HCC, and late-stage HCC

The distributions and comparisons of serum PIVKA-II were further investigated according to the above results of independent factors associated with the abnormal elevation of PIVKA-II level.

According to Figure 2A, the median PIVKA-II levels among subgroups of different etiologies were significantly different ($P < 0.0001$); for viral liver disease without HCC, no difference was observed between HBV and HCV (23.8 *vs* 21.5, $P > 0.05$), but among nonviral liver diseases of NAFLD, ALD and AILD (28.0 *vs* 34.5 *vs* 20.0, $P < 0.0001$), the differences were significant. Further analysis showed that compared to CLD patients with HBV or HCV, the median PIVKA-II level in ALD was substantially higher (both $P < 0.0001$), and also in NAFLD (both $P < 0.05$). Meanwhile, AILD patients had significantly lower PIVKA-II levels than HBV patients ($P < 0.05$), whereas no difference was observed between HCV and AILD ($P > 0.05$). For patients with HCC (Figure 2B), the median PIVKA-II levels gradually significantly increased as the tumor size changed by ≤ 2 cm, > 2 cm and ≤ 5 cm, > 5 cm and ≤ 10 cm to > 10 cm (39.5 *vs* 135.5 *vs* 1811.0 *vs* 30987.0, $P < 0.0001$).

Further analysis revealed that viral liver disease patients had a significantly lower median level of PIVKA-II than patients with nonviral liver disease (23.0 *vs* 27.0, $P < 0.0001$) in non-HCC (Figure 2C), and there were also similar trends between ALP ≤ 1 × ULN and ALP > 1 × ULN (23.0 *vs* 30.0, $P < 0.0001$) (Figure 2D), TBIL ≤ 1 × ULN and TBIL > 1 × ULN (23.0 *vs* 27.0, $P < 0.0001$) (Figure 2E), AST ≤ 1 × ULN and AST > 1 × ULN (23.0 *vs* 26.0, $P < 0.01$) (Figure 2F) in non-HCC. However, similar tendencies were

Table 1 Clinical characteristics of patients with non-hepatocellular carcinoma and hepatocellular carcinoma

Variable	Non-HCC			HCC		
	Viral liver disease (*n* = 595)	Nonviral liver disease (*n* = 189)	*P* value	Early stage HCC (*n* = 127)	Late stage HCC (*n* = 140)	*P* value
Age (yr)	46.2 ± 13.4	49.6 ± 13.5	0.003	56.5 ± 10.8	55.5 ± 12.1	0.488
Male, *n* (%)	418 (70.3)	117 (61.9)	0.032	113 (89.0)	125 (89.3)	0.935
Etiology			-			0.333
HBV	561 (94.3)	-		115 (90.6)	130 (92.9)	
HCV	34 (5.7)	-		2 (1.6)	2 (1.4)	
NAFLD	-	56 (29.6)		2 (1.6)	-	
ALD	-	82 (43.4)		6 (4.7)	8 (5.7)	
AILD	-	51 (27.0)		2 (1.6)	-	
Cirrhosis, *n* (%)	274 (46.1)	75 (39.7)	0.125	112 (88.2)	120 (85.7)	0.550
ALT (U/L)	48 (25, 171)	40 (24, 74)	< 0.001	31 (22, 42)	40 (25, 67)	0.003
AST (U/L)	44 (27, 101)	38 (25, 60)	0.007	32 (24, 47)	60 (35, 95)	< 0.001
ALP (U/L)	92 (75, 122)	97 (72, 141)	0.153	90 (70, 120)	112 (88, 177)	< 0.001
GGT (U/L)	50 (28, 113)	94 (39, 241)	< 0.001	44 (25, 78)	113 (57, 208)	< 0.001
TBIL (μmol/L)	19.8 (13.2, 31.6)	16.1 (10.5, 35.0)	0.006	18.4 (11.6, 29.4)	19.3 (13.6, 30.2)	0.277
ALB (g/L)	38.8 ± 6.3	38.7 ± 7.7	0.834	38.3 ± 7.0	36.6 ± 6.0	0.036
PLT (10^9/L)	161 (104, 199)	206 (124, 273)	< 0.001	145 (87, 178)	188 (132, 242)	< 0.001
Tumor size (cm)	-	-	-	2.2 (1.5, 2.9)	9.3 (5.8, 13.2)	< 0.001
Number of tumors (1/2-3/> 3)	-	-	-	115/12/0	62/33/45	< 0.001
Vascular invasion	-	-	-	-	53 (37.9)	-
Extrahepatic metastases	-	-	-	-	13 (9.3)	-
PIVKA II > 40 mAU/mL, *n* (%)	83 (13.9)	39 (20.6)	0.027	77 (60.6)	131 (93.6)	< 0.001
PIVKA II (mAU/mL)	23.0 (18.0, 31.0)	27.0 (20.0, 38.0)	< 0.001	58.0 (25.0, 228.0)	5124 (691.0, 36245.0)	< 0.001

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; PIVKA-II: Protein induced by vitamin K absence or antagonist-II.

not observed in early-stage HCC patients, and the PIVKA-II levels between the above subgroups did not differ significantly. Furthermore, for late-stage HCC, the median level of PIVKA-II of ALP ≤ 1 × ULN was significantly lower compared to that of ALP > 1 × ULN (2129 *vs* 11992, $P < 0.01$), and also between AST ≤ 1 × ULN and AST > 1 × ULN (593.0 *vs* 15164, $P < 0.0001$); however, between viral and nonviral liver disease, a significant difference was not observed, nor between TBIL ≤ 1 × ULN and TBIL > 1 × ULN.

Additionally, Supplementary Table 1 shows that irrespective of the subgroups of etiology (viral and nonviral liver disease), ALP (≤ 1 × ULN and > 1 × ULN), TBIL (≤ 1 × ULN and > 1 × ULN), AST (≤ 1 × ULN and > 1 × ULN), serum PIVKA-II levels differed significantly among non-HCC, early-stage and late-stage HCC.

Significant influence of serum TBIL on the performance of PIVKA-II in diagnosing early-stage HCC but not for late-stage HCC

The above findings suggested that abnormally elevated PIVKA-II level was independently positively associated with etiology, ALP, and TBIL for non-HCC patients and AST and tumor size for HCC patients. Then, the AUCs of serum PIVKA-II for the diagnosis of early-stage HCC and late-stage HCC were further analyzed for each subgroup of patients by etiology, ALP, TBIL, and AST.

Table 2 Factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analysis in non-hepatocellular carcinoma patients

Non-HCC	Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (yr)	1.009 (0.995, 1.024)	0.195		
Gender (M)	2.102 (1.308, 3.379)	0.002		
Etiology		< 0.001		< 0.001
NAFLD	-		-	
HBV	4.556 (1.089, 19.055)		3.214 (0.762, 13.548)	
HCV	1.687 (0.227, 12.571)		1.528 (0.204, 11.442)	
ALD	15.577 (3.542, 68.507)		9.883 (2.216, 44.086)	
AILD	4.295 (0.849, 21.729)		1.993 (0.368, 10.792)	
Cirrhosis (+)	1.848 (1.250, 2.731)	0.002		
ALT/ULN	1.026 (0.991, 1.063)	0.150		
AST/ULN	1.076 (1.029, 1.126)	0.001		
ALP/ULN	2.630 (1.823, 3.795)	< 0.001	2.146 (1.429, 3.221)	< 0.001
GGT/ULN	1.105 (1.050, 1.163)	< 0.001		
TBIL/ULN	1.228 (1.142, 1.321)	< 0.001	1.162 (1.080, 1.250)	< 0.001
ALB/LLN	0.063 (0.024, 0.166)	< 0.001		
PLT/LLN	0.618 (0.445, 0.857)	0.004		

HCC: Hepatocellular carcinoma; OR: Odds ratio; CI: Confidence interval; M: male; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; ULN: Upper limit of normal; LLN: Lower limit of normal.

In Table 4, serum PIVKA-II had the best performance for diagnosing early-stage HCC in TBIL ≤ 1 × ULN subgroup, and the AUC of PIVKA-II in TBIL ≤ 1 × ULN subgroup was significantly higher than that in TBIL > 1 × ULN subgroup (0.817 *vs* 0.669, $P = 0.015$). When compared with the subgroup of nonviral liver disease, the AUC of PIVKA-II in the subgroup of viral liver disease for diagnosing early-stage HCC was only marginally higher (0.783 *vs* 0.736, $P = 0.678$), the difference was not significant, nor was the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN (0.783 *vs* 0.729, $P = 0.398$). However, the AUC of PIVKA-II in AST ≤ 1 × ULN subgroup was almost the same to AST > 1 × ULN subgroup (0.774 *vs* 0.778, $P = 0.941$) in diagnosing early-stage HCC. Figure 3 shows the corresponding ROC curves of PIVKA-II for diagnosing early-stage HCC in different subgroups.

However, subsequent analysis of Supplementary Table 2 showed that the above factors, including etiology, ALP, TBIL, and AST, had almost no influence on the performance of PIVKA-II for the diagnosis of late-stage HCC. The AUCs of PIVKA-II did not differ significantly between subgroups in etiology (viral *vs* nonviral), ALP (≤ 1 × ULN *vs* > 1 × ULN), TBIL (≤ 1 × ULN *vs* > 1 × ULN) and AST (≤ 1 × ULN *vs* > 1 × ULN) (P all > 0.05). Supplementary Figure 2 also shows the corresponding ROC curves of PIVKA-II for diagnosing late-stage HCC in different subgroups.

Furthermore, we also analyzed the AUCs of PIVKA-II for HCC between viral and ALD etiologies. Supplementary Figure 3 shows that compared to the subgroup of viral etiology, the AUC of PIVKA-II for early-stage HCC was lower in ALD etiology (0.783 *vs* 0.655, $P = 0.470$), and also for late-stage HCC (0.970 *vs* 0.909, $P = 0.361$).

Serum PIVKA-II had better performance in diagnosing early-stage HCC for patients with TBIL ≤ 1 × ULN, both of viral etiology and HBV alone

Different etiologies of liver disease might induce diverse progression of liver injuries and changes in indicators of liver function, such as serum ALP and TBIL. Then, we further analyzed the AUCs of ROC curves of PIVKA-II by each subgroup of ALP and TBIL in patients of viral etiology and HBV alone, respectively, but not in patients with nonviral etiology on account of the limited cases of HCC.

Figure 4 shows that patients with TBIL ≤ 1 × ULN of viral etiology had substantially higher AUC of PIVKA-II for diagnosing early-stage HCC (0.837 *vs* 0.677, $P = 0.012$) than patients with TBIL > 1ULN.

Table 3 Factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analyses in hepatocellular carcinoma patients

HCC	Univariate		Multivariate	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (yr)	1.002 (0.977, 1.027)	0.895		
Gender (M)	1.692 (0.726, 3.944)	0.223		
Etiology (viral)	1.008 (0.319, 3.185)	0.989		
Cirrhosis (+)	0.712 (0.280, 1.810)	0.475		
ALT/ULN	2.242 (1.235, 4.068)	0.008		
AST/ULN	2.745 (1.573, 4.788)	< 0.001	1.759 (1.072, 2.887)	0.025
ALP/ULN	3.542 (1.478, 8.490)	0.005		
GGT/ULN	1.436 (1.120, 1.843)	0.004		
TBIL/ULN	1.287 (0.947, 1.749)	0.108		
ALB/LLN	0.202 (0.042, 0.974)	0.046		
PLT/LLN	1.558 (0.985, 2.464)	0.058		
Tumor size (cm)	1.394 (1.221, 1.592)	< 0.001	1.349 (1.175, 1.549)	< 0.001
Number of tumors (1/2-3/> 3)	2.242 (1.340, 3.752)	0.002		
Vascular invasion (+)	5.907 (1.772, 19.696)	0.004		
Extrahepatic metastases (+)	3.551 (0.452, 27.887)	0.228		

HCC: Hepatocellular carcinoma; OR: Odds ratio; CI: Confidence interval; M: Male; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltranspeptidase; TBIL: Total bilirubin; ALB: Albumin; PLT: Platelet; ULN: Upper limit of normal; LLN: Lower limit of normal.

Also, for patients with viral etiology, the AUC of PIVKA-II in ALP ≤ 1 × ULN was higher (0.800 *vs* 0.731, $P = 0.318$) than that in ALP > 1 × ULN, but no statistical significance was found. Supplementary Table 3 further shows the detailed value of AUC, best cutoff value, sensitivity, specificity, LR+, and LR- in each subgroup.

Furthermore, similar tendencies were also validated in patients with HBV alone. As shown in Supplementary Table 4, for patients with HBV alone, the AUCs of PIVKA-II were 0.832 and 0.676 ($P = 0.015$) in TBIL ≤ 1 × ULN and TBIL > 1 × ULN, and 0.794 and 0.732 ($P = 0.368$) in ALP ≤ 1 × ULN and ALP > 1 × ULN, respectively.

When the cutoff value of PIVKA-II for detecting early-stage HCC was set at 40 mAU/mL (ULN), the sensitivity, specificity, LR+, and LR- were further analyzed in patients with viral etiology and HBV alone. As shown in Table 5, compared to TBIL > 1 × ULN of viral etiology, the sensitivity of PIVKA-II in TBIL ≤ 1 × ULN of viral etiology increased from 30.30% to 57.47%, and the LR+ increased from 3.80 to 6.66, without compromising the corresponding specificity (92.02% *vs* 91.37%). Similarly, for patients with HBV alone, the sensitivity of PIVKA-II in TBIL ≤ 1 × ULN was also significantly higher than that in TBIL > 1 × ULN (57.14% *vs* 30.77%), as well as LR+ (6.16 *vs* 3.73), with similar specificity (90.72% *vs* 91.76%). Noticeably, the sensitivities in TBIL ≤ 1 × ULN have consistently been greater than those in ALP≤ 1 × ULN, both for patients with viral etiology (57.47% *vs* 52.88%) and HBV alone (57.14% *vs* 53.00%), and the corresponding specificities were almost the same. However, this tendency disappeared, and the sensitivities tended to be the same between TBIL > 1 × ULN and ALP > 1 × ULN, regardless of viral etiology (30.30% *vs* 30.61%) and HBV alone (30.77% *vs* 30.61%).

DISCUSSION

In the current study, 1051 patients in total were analyzed, and we determined the independent variables linked to elevated serum PIVKA-II levels in CLD and HCC patients, with the purpose of expounding their influence on the PIVKA-II performance for early-stage HCC and late-stage HCC detection. For patients with CLD, abnormally increased PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL, but serum AST and tumor size for HCC patients. Compared to subgroups of nonviral etiology, ALP > 1 × ULN, TBIL > 1 × ULN and AST > 1 × ULN, serum PIVKA-II levels were significantly lower in subgroups of viral etiology, ALP ≤ 1 × ULN, TBIL ≤ 1 × ULN, and

Table 4 Performance characteristics of serum protein induced by vitamin K absence or antagonist-II in diagnosing early-stage hepatocellular carcinoma in different subgroups

Early HCC	AUC (95%CI)	Cutoff value (mAU/mL)	Se (%)	Sp (%)	LR+	LR-	*P* value
All early	0.765 (0.714, 0.815)	41.0	60.63	85.08	4.06	0.46	
Etiology							0.678
Viral	0.783 (0.732, 0.835)	42.8	58.97	87.73	4.81	0.47	
Nonviral	0.736 (0.519, 0.952)	177.0	60.00	94.18	10.31	0.42	
ALP							0.398
≤ 1 ULN	0.783 (0.725, 0.840)	41.0	58.42	89.95	5.81	0.46	
> 1 ULN	0.729 (0.618, 0.841)	42.0	69.23	71.07	2.39	0.43	
TBIL							0.015
≤ 1 ULN	0.817 (0.762, 0.872)	41.0	60.67	91.73	7.34	0.43	
> 1 ULN	0.669 (0.563, 0.775)	42.0	60.53	73.19	2.26	0.54	
AST							0.941
≤ 1 ULN	0.774 (0.712, 0.836)	41.0	57.95	88.08	4.86	0.48	
> 1 ULN	0.778 (0.691, 0.864)	41.0	66.67	82.41	3.79	0.40	

AUC: Area under the ROC curve; CI: Confidence interval; Se: Sensitivity; Sp: Specificity; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; ALP: Alkaline phosphatase; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ULN: Upper limit of normal.

Table 5 Sensitivity, specificity, positive/negative likelihood ratio of protein induced by vitamin K absence or antagonist-II in diagnosing early-stage hepatocellular carcinoma at the cutoff value of 40 mAU/mL in patients with viral etiology and hepatitis B virus alone, respectively

	Cutoff value (mAU/mL)	Viral etiology				HBV			
		Se (%)	Sp (%)	+LR	-LR	Se (%)	Sp (%)	+LR	-LR
ALP									
≤ 1ULN	40	52.88	91.39	6.14	0.52	53.00	90.80	5.76	0.52
1ULN	40	30.61	92.45	4.06	0.75	30.61	92.23	3.94	0.75
TBIL									
≤ 1ULN	40	57.47	91.37	6.66	0.47	57.14	90.72	6.16	0.47
1ULN	40	30.30	92.02	3.80	0.76	30.77	91.76	3.73	0.75

HBV: Hepatitis B virus; Se: Sensitivity; Sp: Specificity; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; ALP: Alkaline phosphatase; TBIL: Total bilirubin; ULN: Upper limit of normal.

AST ≤ 1 × ULN in CLD patients; however, these differences disappeared in early-stage HCC. Furthermore, serum PIVKA-II in a subgroup of TBIL ≤ 1 × ULN always had the highest AUCs and the best performance in detecting early-stage HCC than TBIL > 1 × ULN, irrespective of all patients, viral etiology or HBV alone. However, the above factors, including etiology, ALP, TBIL, and AST, had almost no influence on PIVKA-II performance for late-stage HCC detection.

Four previous studies found that ALD patients had higher levels of PIVKA-II compared to viral hepatitis-related CLD patients[23-26], and ALD was also verified to be a significant factor related to positive serum PIVKA-II by a retrospective case-control study[26]. Consistently, in this study, patients with CLD with ALD etiology had the highest median level of PIVKA-II compared to those with other etiologies of HBV, HCV, NAFLD, and AILD, and was demonstrated to be independently associated with abnormally elevated PIVKA-II levels. A recent study enrolled 130 cases and showed that patients with CHB, CHC, and nonviral CLD had no significant differences in PIVKA-II levels (32 mAU/mL *vs* 35 mAU/mL *vs* 35 mAU/mL, any two $P > 0.05$)[31]. In the current study, however, a significant difference was observed in PIVKA-II levels between viral and nonviral CLD patients; meanwhile, PIVKA-II levels in NAFLD and ALD patients were significantly higher compared to those in HBV and HCV patients,

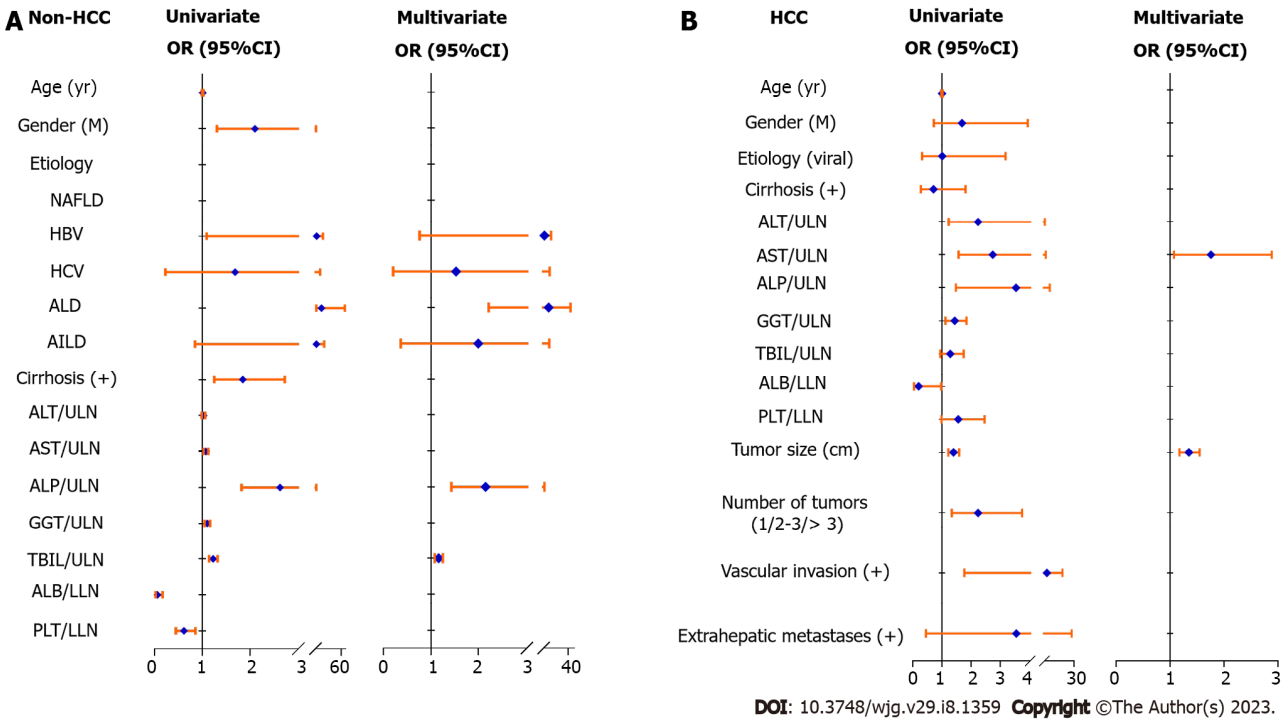

Figure 1 Forest plots of factors associated with abnormally elevated protein induced by vitamin K absence or antagonist-II by univariate and multivariate logistic analysis in patients with non-hepatocellular carcinoma and hepatocellular carcinoma. A: In non- hepatocellular carcinoma (HCC) patients; B: In HCC patients. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ULN: Upper limit of normal; TBIL: Total bilirubin; PLT: Platelet; LLN: Lower limit of normal; 95%CI: 95% confidence interval; OR: Odds ratio.

and AILD patients had the lowest PIVKA-II levels than those of other CLD patients.

In this study, tumor size was an independent factor relevant to abnormally elevated PIVKA-II levels in HCC patients, and the median levels of PIVKA-II gradually significantly increased as the tumor size changed by ≤ 2 cm, > 2 cm and ≤ 5 cm, > 5 cm and ≤ 10 cm to > 10 cm, which was consistent with the results of previous studies[32,33]. Moreover, the number of tumors and vascular invasion were associated with the abnormal elevation of PIVKA-II level which was only perceived in the univariate analysis. The results imply that tumor size had more influence on the PIVKA-II level than the number of tumors and vascular invasion.

Prior researches had reported that PIVKA-II serum levels are not elevated in hepatic flares and are influenced by liver regeneration triggered by necroinflammation in patients with active CLD, and have paid little attention to the confounding factors of liver injury and function that influence the levels of PIVKA-II[12,31]. Nonetheless, this is the first study to our knowledge to demonstrate that the abnormal PIVKA-II levels are significantly associated with serum ALP and TBIL in CLD patients and serum AST in HCC patients. Furthermore, in patients with CLD, the median PIVKA-II levels differed significantly between the subgroups of ALP ≤ 1 × ULN and ALP > 1 × ULN, as well as between TBIL ≤ 1 × ULN and TBIL > 1 × ULN; and in patients with late-stage HCC, the differences in PIVKA-II median levels were also observed between the subgroups of ALP and AST classified by 1 × ULN. However, these changes in PIVKA-II serum levels disappeared in early-stage HCC patients in the same situation.

Regretfully, the underlying mechanism behind the associations of ALD, ALP, and TBIL with elevated PIVKA-II levels in CLD patients remains unclear. Although vitamin K insufficiency may arise in chronic alcoholics[34], prior studies[23,25] found there was no direct correlation between PIVKA-II serum levels and vitamin K serum concentration. For ALP and TBIL, one likely explanation is that abnormalities in serum ALP and TBIL were always seen in all types of liver disorders and cholestasis. Vitamin K deficiency, as one of the fat-soluble vitamin deficiencies, is also a typical complication in chronic cholestasis patients, and vitamin levels were inversely correlated with serum TBIL levels[35-37]. Interestingly, one recent study also reported that hepatitis E patients in the raised PIVKA-II subgroup had significantly greater TBIL levels than those in the normal PIVKA-II subgroup ($P < 0.05$), and the trend of changes in serum PIVKA and TBIL were similar and related to the disease course[38].

It has been reported that the PIVKA-II's AUC for HCC in the CHB group was the highest compared to those in CHC and nonviral CLD patients (0.833 *vs* 0.732 *vs* 0.806)[31]. Similarly, in the current study, the AUC of PIVKA-II was slightly higher in patients with viral etiology compared to that in patients with nonviral etiology, irrespective of whether the HCC is in its early or late stages. Patients with ALD etiology had much lower AUCs of PIVKA-II for detecting HCC than patients with viral etiology,

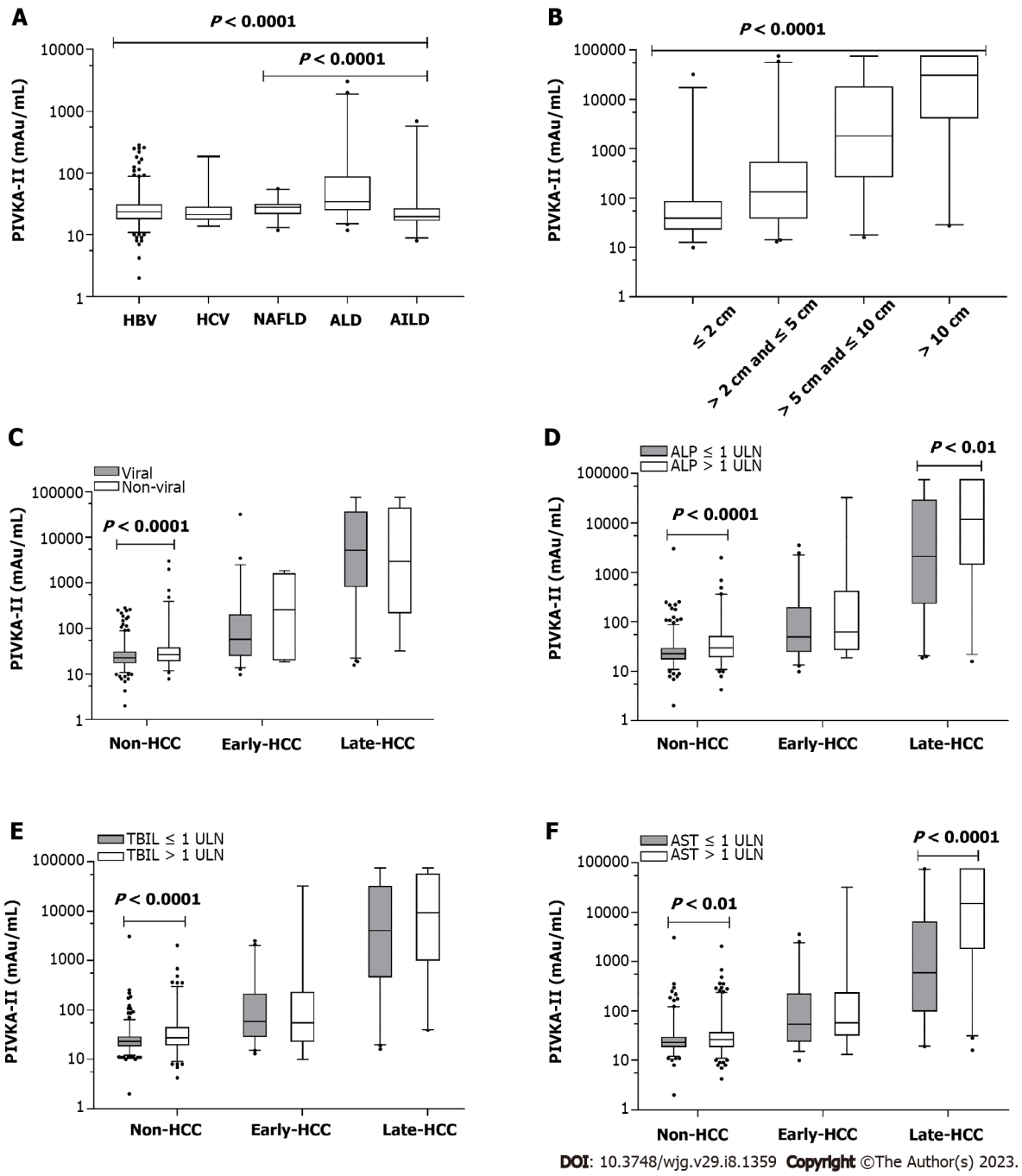

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Figure 2 Distributions and comparisons of serum protein induced by vitamin K absence or antagonist-II in different subgroups. A: Different etiologies of hepatitis B virus (HBV), HCV, nonalcoholic fatty liver disease, alcohol-related liver disease (ALD), autoimmune liver disease; B: Different tumor sizes of ≤ 2 cm (n = 60), > 2 cm and ≤ 5 cm (n = 88), > 5 cm and ≤ 10 cm (n = 65), > 10 cm (n = 54); C: Subgroups between viral and nonviral liver diseases; D: Subgroups between alkaline phosphatase (ALP) ≤ 1 × upper limit of normal (ULN) and ALP > 1 × ULN; E: Subgroups between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN; F: Subgroups between aspartate aminotransferase (AST) ≤ 1 × ULN and AST > 1 × ULN. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; ALD: Alcohol-related liver disease; AILD: Autoimmune liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ULN: Upper limit of normal; TBIL: Total bilirubin.

especially for early-stage HCC. However, in previous literature, the influences of serum ALP and TBIL on the performance of PIVKA-II for the detection of HCC have not been evaluated. In this study, we provided evidence that patients in TBIL ≤ 1 × ULN subgroup had the best performance of PIVKA-II for detecting early-stage HCC, and the AUC of PIVKA-II in TBIL ≤ 1 × ULN subgroup was significantly higher compared to that in TBIL > 1 × ULN subgroup. Between ALP ≤ 1 × ULN and ALP > 1 × ULN, a similar trend was also observed, although the difference was not significant. However, no significant influences of serum ALP and TBIL were observed on the performance of PIVKA-II for late-stage HCC.

Further analysis also showed that the above changes and differences in the performance of PIVKA-II for the detection of early-stage HCC still existed between different subgroups of ALP and TBIL in patients with viral etiology and HBV alone. Moreover, when 40 mAU/mL (ULN) was set as the cutoff

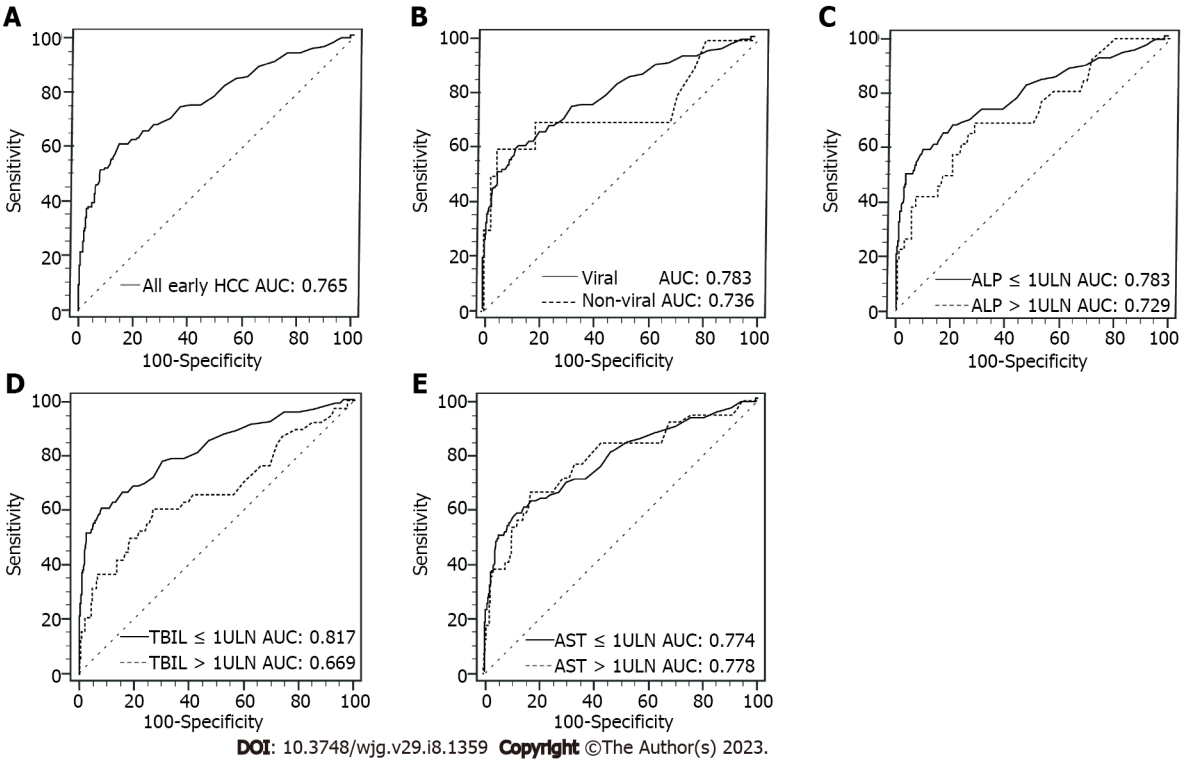

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Figure 3 Comparisons of receiver operating characteristic curves of serum protein induced by vitamin K absence or antagonist-II for diagnosing early-stage hepatocellular carcinoma in different subgroups. A: For all early-stage patients; B: Between viral and nonviral subgroups; C: Between alkaline phosphatase ≤ 1 × upper limit of normal (ULN) and alkaline phosphatase > 1 × ULN subgroups; D: Between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN subgroups; E: Between aspartate aminotransferase (AST) ≤ 1 × ULN and AST > 1 × ULN subgroups. ULN: Upper limit of normal; TBIL: Total bilirubin; HCC: Hepatocellular carcinoma; AUC: Area under the receiver operating characteristic curve; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

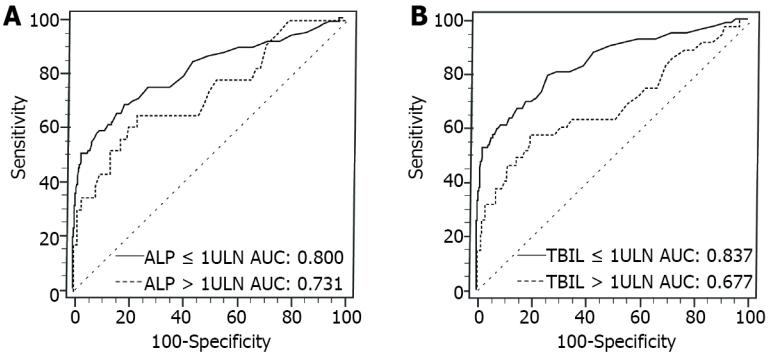

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Figure 4 Comparisons of receiver operating characteristic curves of serum protein induced by vitamin K absence or antagonist-II for diagnosing early-stage hepatocellular carcinoma in subgroups of alkaline phosphatase and total bilirubin for patients with viral etiology. A: Between alkaline phosphatase (ALP) ≤ 1 × upper limit of normal (ULN) and ALP > 1 × ULN subgroups; B: Between total bilirubin (TBIL) ≤ 1 × ULN and TBIL > 1 × ULN subgroups. ULN: Upper limit of normal; TBIL: Total bilirubin; ALP: Alkaline phosphatase; AUC: Area under the receiver operating characteristic curve.

value of PIVKA-II, for patients with TBIL ≤ 1 × ULN, serum PIVKA-II had the highest sensitivities in detecting early-stage HCC than other subgroups and enough high specificities at the same time, irrespective of viral etiology or HBV alone. These results strongly suggest that serum PIVKA-II would have better performance in detecting HCC at an early-stage for patients with normal serum TBIL.

The current study had a number of limitations. Firstly, it was a retrospective design, and some unidentified potential biases might exist, although we completely ruled out the possible interferences on serum PIVKA-II by the potential confounding factors in the exclusion criteria. Then, the limited sample size, including HCC patients with CHC, ALD, NAFLD, and AILD, does not allow us to independently evaluate the influences of these factors on the performance of PIVKA-II in HCC patients with CHC, NAFLD, and AILD, and decrease the reliability of that in HCC patients with ALD. Finally, further

multicenter research with more participants are required to validate the above findings.

CONCLUSION

The present study suggests that abnormally elevated PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL for non-HCC CLD patients and serum AST and tumor size for HCC patients. Better performance of PIVKA-II for discriminating HCC at an early stage from patients with CLD would be achieved in patients with normal TBIL, and more attention should be given to the availability of PIVKA-II in HCC surveillance for at-risk patients with elevated serum TBIL.

ARTICLE HIGHLIGHTS

Research background

Serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) is a promising biomarker for hepatocellular carcinoma (HCC) surveillance.

Research motivation

Investigate those unclear factors regarding different liver disease etiologies, the liver injury severity, and disease activity associated with the abnormal levels of serum PIVKA-II in chronic liver disease (CLD) and HCC patients.

Research objectives

Identify the potential contributing factors and evaluate their influence on serum PIVKA-II levels and the performance of PIVKA-II for the diagnosis of HCC in different populations.

Research methods

This study retrospectively enrolled in 784 CLD patients and 267 HCC patients in Mengchao Hepatobiliary Hospital of Fujian Medical University from April 2016 to December 2019. Logistic regression and the area under the receiver operating characteristic curve (AUC) were used to evaluate the influencing factors and diagnostic performance of PIVKA-II for HCC, respectively.

Research results

Elevated PIVKA-II levels were independently positively associated with alcohol-related liver disease (ALD), serum alkaline phosphatase (ALP), and total bilirubin (TBIL) for CLD patients and aspartate aminotransferase (AST) and tumor size for HCC patients (all $P < 0.05$). Serum PIVKA-II were significantly lower in patients with viral etiology, ALP ≤ 1 × upper limit of normal (ULN), TBIL ≤ 1 × ULN, and AST ≤ 1 × ULN than in those with nonviral disease and abnormal ALP, TBIL, or AST (all $P < 0.05$), but the differences disappeared in patients with early-stage HCC. For patients with TBIL ≤ 1 × ULN, the AUC of PIVKA-II was significantly higher compared to that in patients with TBIL > 1 × ULN (0.817 *vs* 0.669, $P = 0.015$), while the difference between ALP ≤ 1 × ULN and ALP > 1 × ULN was not statistically significant found between ALP ≤ 1 × ULN and ALP > 1 × ULN (0.783 *vs* 0.729, $P = 0.398$). These trends were then more prominently perceived in subgroups of patients with viral etiology and HBV alone.

Research conclusions

Abnormally elevated PIVKA-II levels were independently positively associated with ALD etiology, serum ALP and TBIL for non-HCC CLD patients and serum AST and tumor size for HCC patients. Better performance of PIVKA-II for discriminating HCC at an early stage from patients with CLD would be achieved in patients with normal TBIL.

Research perspectives

More attention should be given to the availability of PIVKA-II in HCC surveillance for at-risk patients with elevated serum TBIL, which may be important for surveillance counseling of early-stage HCC.

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FOOTNOTES

Author contributions: Qian XJ, Wen ZM, and Huang XM contributed to acquisition of data, analysis and interpretation of data, drafting of the manuscript, and statistical analysis; Feng HJ, Li SC, Zhang Y, Peng WG, Yang JR, Zheng ZY, and Zhang L contributed to acquisition of data and critical revision of the manuscript for important intellectual content; Lin SS, Liu YN, and Zhang DW contributed to administrative, technical, material support, and critical revision of the manuscript for important intellectual content; Lu FM, Liu LJ, and Pan WD contributed to study concept and design, obtained funding and material support, study supervision and critical revision of the manuscript for important intellectual content; all authors of this research have approved the final version of the article; Qian XJ, Wen ZM, and Huang XM contributed equally to this work; Lu FM, Liu LJ, and Pan WD contributed equally to this work.

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Country/Territory of origin: China

ORCID number: Xiang-Jun Qian 0000-0001-6485-0731; Feng-Min Lu 0000-0002-1832-3209; Wei-Dong Pan 0000-0001-9519-7683.

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SYSTEMATIC REVIEWS

Burden of bone disease in chronic pancreatitis: A systematic review and meta-analysis

Ankit Chhoda, Maria Jose Hernandez-Woodbine, Nana Afua Akkya Addo, Syed Alishan Nasir, Alyssa Grimshaw, Craig Gunderson, Awais Ahmed, Steven D. Freedman, Sunil G. Sheth

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Ankit Chhoda, Maria Jose Hernandez-Woodbine, Awais Ahmed, Steven D. Freedman, Sunil G. Sheth, Department of Gastroenterology, Beth Israel Deaconess Medical Center, Boston, MA 02215, United States

Nana Afua Akkya Addo, Syed Alishan Nasir, Department of Medicine, Norwalk Hospital, Yale School of Medicine, Norwalk, CT 06850, United States

Alyssa Grimshaw, Cushing/Whitney Medical Library, Yale University, New Haven, CT 06510, United States

Craig Gunderson, General Internal Medicine, Yale School of Medicine, New Haven, CT 06510, United States

Corresponding author: Sunil G. Sheth, AGAF, FACG, FASGE, MBBS, MD, Associate Professor, Department of Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, United States. ssheth@bidmc.havard.edu

Abstract

BACKGROUND

Bone disease is an under-recognized cause of morbidity in chronic pancreatitis (CP). Over the past decade, publications of original studies on bone disease in CP has warranted synthesis of the evidence to ascertain the true burden of the problem.

AIM

To quantify the prevalence of osteopenia, osteoporosis, and fragility fractures in CP patients and investigate the associated clinical features and outcomes.

METHODS

A systematic search identified studies investigating bone disease in CP patients from Cochrane Library, Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science, from inception until October 2022. The outcomes included prevalence of osteopenia, osteoporosis, and fragility fractures, which were meta-analyzed using a random-effects model and underwent metaregression to delineate association with baseline clinical features.

RESULTS

Twenty-one studies were included for systematic review and 18 studies were

included for meta-analysis. The pooled prevalence of osteopenia and osteoporosis in CP patients was 41.2% (95%CI: 35.2%-47.3%) and 20.9% (95%CI: 14.9%-27.6%), respectively. The pooled prevalence of fragility fractures described among CP was 5.9% (95%CI: 3.9%-8.4%). Meta-regression revealed significant association of pancreatic enzyme replacement therapy (PERT) use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$]. We observed no associations with mean age, sex distribution, body mass index, alcohol or smoking exposure, diabetes with prevalence of osteopenia, osteoporosis or fragility fractures. Paucity of data on systemic inflammation, CP severity, and bone mineralization parameters precluded a formal meta-analysis.

CONCLUSION

This meta-analysis confirms significant bone disease in patients with CP. Other than PERT use, we observed no patient or study-specific factor to be significantly associated with CP-related bone disease. Further studies are needed to identify confounders, at-risk population, and to understand the mechanisms of CP-related bone disease and the implications of treatment response.

Key Words: Chronic pancreatitis; Fractures; Osteoporosis; Osteopenia; Bone disease

Core Tip: Bone disease is an under recognized cause of morbidity in chronic pancreatitis (CP). This systematic review and meta-analysis demonstrate substantial burden of bone disease (osteopenia and osteoporosis) and fragility fractures in CP patients. In addition, metaregression has demonstrated a significant association of osteoporosis with pancreatic enzyme replacement. The study is powered by high-quality studies with large sample size and clearly defined study population and outcome measures.

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive, multifactorial fibro-inflammatory syndrome, which arises from persistent pathological response to noxious stimuli[1]. It is characterized by chronic abdominal pain, exocrine and endocrine insufficiency. Over time, nutritional deficiencies, systemic inflammation, and etiological factors like alcohol or smoking may disrupt the balance between bone formation and resorption[2]. These maladaptive alterations may subsequently cause bone disease *i.e.*, osteopenia and osteoporosis, leading to fractures which result from low energy trauma, called fragility fractures, and have significant implications on quality of life[3].

Osteoporosis is a public health hazard with a substantial economic burden[4]. Recent data from the National Health and Nutrition Examination Survey found its age-adjusted prevalence among adults older than 50 years to be 12.6% affecting more women than men (19.6% *vs* 4.4%). In view of the burden of bone disease and subsequent fracture risk in individuals affected by CP, various consensus guidelines have recommended baseline bone health assessment[5,6]. The adherence to guidelines has been low and studies have demonstrated that less than a quarter of CP patients are screened for bone disease, suggesting that bone disease is an underappreciated source of morbidity in CP[7,8].

A previous systematic review by Duggan *et al*[9] estimated the prevalence of osteopenia and osteoporosis in patients with CP to be 39.8% and 23.4%, respectively. The analysis was based on a limited number of studies ($n = 11$) and did not quantify fragility fractures which are complications of CP-related bone disease. Over the last decade, multiple large-sized studies including those from multicenter cohorts have investigated bone disease in CP as well as the nutritional, anthropometric, and inflammatory parameters which may impact bone health outcomes. Thus, with quantitative data on covariates and fragility fractures available, an updated synthesis of evidence is pertinent. This systematic review and meta-analysis aims to quantify the prevalence of osteopenia, osteoporosis, and fragility fractures in CP patients and to delineate clinical parameters which impact their occurrence.

MATERIALS AND METHODS

Protocol registration

This systematic review was performed in accordance with PRISMA guidelines[10]. The review methodology was pre-registered at open-source forum[11].

Search strategy

A systematic search of the literature was conducted by a medical librarian in the following databases: Cochrane Library, Ovid Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science Core Collection to find articles published from the inception of the database to October 20, 2022. The search was performed using a combination of controlled and free text terms for CP and bone diseases (strategy outlined in Supplementary Table 1). The search was not limited to publication type or date. the search was peer-reviewed by a second librarian using peer review for electronic search strategies[12]. Citations were imported into an Endnote 20 Library (Version 20.2, Clarivate Analytics, Philadelphia), and after removal of duplicates, the remaining studies were imported into Covidence (Melbourne, Australia) for screening and data extraction.

Study selection

Two co-authors (MJ, AC) independently used the inclusion and exclusion criteria to assess titles and abstracts. Eligibility decisions and disagreements were reconciled through discussion with the senior author (SS). Full-text articles included by two reviewers underwent data extraction and quality assessment.

Inclusion criteria: This systematic review included original cross-sectional and cohort studies, which were of prospective and retrospective design. CP patients were diagnosed based on specific codes related to CP per the International Classification of Diseases (ICD-9/10) and through predefined clinical, radiologic and/or histologic findings (described in Table 1).

Exclusion criteria: The studies which did not report bone diseases in CP patients were ineligible for the systematic review. We also excluded studies published in foreign languages, associated with animal research, reviews, abstracts, letters, case reports, and series with a sample size of less than 10 patients were excluded (Figure 1).

Data extraction

We noted data on study design, population characteristics such as age, gender, method of CP diagnosis (ICD-code based *vs* systematic clinical-radiologic features), body mass index (BMI), and exposure to alcohol and smoking. These data were independently extracted by two authors (MJ and AC). The outcome of interest included the prevalence of osteopenia, osteoporosis, and fragility fractures among the CP patients. The definitions of these bone outcome measures were also noted and have been outlined in Table 2.

Meta-analysis methodology

Prevalence rates were pooled using random-effects meta-analysis and calculated using the score method [13]. Between-study heterogeneity was estimated using I^2 statistics wherein 25%, 50%, and 75% were considered to indicate low, moderate, and high heterogeneity, respectively. Sensitivity analysis was conducted by sequentially removing individual studies. Statistical analysis was performed using Stata/IC, version 17 (StataCorp, College Station, TX). Metaregression, subgroup analysis and publication bias assessment could not be performed for analyses including < 10 studies[14].

Metaregression

Metaregression using the restricted maximum likelihood method was also performed using variables of mean age, sex, BMI, smoking or alcohol exposure, diabetes, serum parathyroid hormone (PTH), percentage population with vitamin D deficiency, and pancreatic enzyme replacement therapy (PERT).

Study quality and publication bias assessment

The quality of observational studies was independently assessed using the Newcastle-Ottawa Scale by two investigators (SN and AA) and recorded in a Microsoft Excel spreadsheet (XP Professional Edition; Microsoft Corp, Redmond, WA, United States)[15]. Any discrepancy was resolved by the senior author (SS). Publication bias were assessed by inspection of a funnel plot and Egger's test[16].

Table 1 Description of study design and study population

Ref.	Design	CP patients	CP severity, *n* (%)	CP etiology, *n* (%)	Study population	Race	Female patients, *n* (%)	Age, mean ± SD	BMI, mean ± SD
Morán *et al*[17], 1997	Cross sectional	Clinicoradiological	All severe	Alcohol: 10 (71.4)	CP: 14		CP: 0	CP: 56[1] (-)	CP: 22.64
				Idiopathic 4 (28.6)	Controls: -		Controls: -	Controls: -	Controls: -
Haaber *et al*[18], 2000	Cross sectional	Clinicoradiological		Alcohol: 46 (79)	CP: 58	CP: -	CP: 26 (44.8)	CP: 53 (9)	CP: 23 (5)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Dujsikova *et al* [19], 2008	Cross sectional	EUS based criteria	Wiersema classification: Mild: 41 (56.2), moderate: 12 (16.4), severe: 20 (27.4)		CP: 73	CP: -	CP:17 (23.28)	CP: 46.61 (13.23)	CP: -
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Tignor *et al*[20], 2010	Retrospective cohort	ICD-9 code 577.1			CP: 3192	CP: White: 2091 (65.5), black: 419 (13.1), hispanic: 222 (6.9), others: 532 (16.7)	CP: 1636 (51.25)	CP: -	CP: -
					Controls: 1436699	Control: White: 860190 (59.9), black: 115199 (8.0), hispanic: 102000, other: 451110	Controls: 907328 (63.15)	Controls: -	Controls: -
Sudeep *et al*[22], 2011	Cross sectional	Not defined		Tropical pancreatitis: 20 (65)	CP: 31	CP: -	CP: 0	CP: 35.8 (9)	CP: 18.46 (2.86)
				Idiopathic: 11 (35)	Controls: 35	Controls: -	Controls: 0	Controls: 38.6 (5.2)	Controls: 22.6 (3.1)
Joshi *et al*[21], 2011	Cross sectional	Clinicoradiological		All patients with tropical calcific pancreatitis	CP: 72	CP: -	CP: 34 (47.2)	CP: 31.1 (10.3)	CP: 19 (3.1)
					Controls: 100	Controls: -	Controls: 50 (50)	Controls: 32.6 (9.6)	Controls: 23.6 (3.2)
Duggan *et al* [23], 2012	Cross sectional	Clinicoradiological	Cambridge classification: Mild (37.1), severe (27.4)	Alcohol: 24 (38.7)	CP: 62	CP: -	CP: 17 (27.41)	CP: 47.9 (12.5)	CP: 25.6 (5)
				Idiopathic: 38 (61.3)	Controls: 66	Controls: -	Controls: 18 (27.27)	Controls: 47.74 (11)	Controls: 28.0 (4.1)
Sikkens *et al*[25], 2013	Prospective cohort	Clinicoradiological		Alcohol: 20 (50)	CP: 40	CP: -	CP: 17 (42.5)	CP: 52 (11)	CP: 24 (5)
				Idiopathic: 17 (43)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Other: 3 (7)					
Prabhakaran *et al* [28], 2014	Cross sectional	Clinicoradiological.	Cambridge classification: Mild (13.1), moderate (5.05), marked: (81.8)	Alcohol: 72 (70)	CP: 103	CP: -	CP: 0	CP: 38.6 (20.64)	CP: 19.7

				Idiopathic: 31 (29.1)	Controls: -	Controls: -	Controls: 0	Controls: 36.7 (20.70)	Controls: -
Bang *et al*[26], 2014	Prospective cohort	ICD-10: K86.0 (alcohol induced CP), K86.1 (other CP)			CP: 11972	CP: -	CP: 4011 (33.5)	CP: 54.5 (14)	CP: -
					Controls: 119720	Controls: -	Controls: 40106 (33.49)	Controls: 54.5 (14)	Controls: -
Duggan *et al* [27], 2015	Cross sectional	Clinicoradiological	Cambridge classification (unspecified number in each category)	Alcohol: 18 (62.1)	CP: 29	CP: -	CP: 12 (41.37)	CP: 44.3 (12.3)	CP: 25.2 (5.1)
				Idiopathic: 8 (27.6)	Controls: 29	Controls: -	Controls: 12 (41.37)	Controls: 45.8 (9.8)	Controls: 27.3 (3.7)
				Other: 3 (10.3)					
Munigala *et al* [24], 2016	Cross sectional	ICD-9 code 577.1			CP: 3257	CP: White 2120 (65), black 1012 (31), others 125 (4)	CP: 178 (5.46)	CP: 54.2 (11.1)	CP: -
					Controls: 450655	Controls: White: 325132 (72), black: 76031 (17), others: 49492 (11)	Controls: 53108 (11.78)	Controls: 53.6 (13.9)	Controls: -
Kumar *et al*[29], 2017	Cross sectional	Clinicoradiological			CP: 102	CP: -	CP: 17 (16.7)	CP: 40.8 (12.6)	CP: 22.5 (3.2)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Stigliano *et al* [32], 2018	Cross sectional	M-ANNHEIM criteria	M-ANNHEIM scoring system: Minor: 74 (35), Increased: 99 (47), advanced: 32 (15), marked: 6 (3)	Alcoholic: 91 (43)	CP: 211	CP: -	CP: 69 (32.7)	CP: 60 (-)	CP: 24 (4)
				Idiopathic: 40 (19)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Hereditary: 8 (4)					
				Obstructive: 12 (5.7)					
Kuhlmann *et al* [30], 2018	Cross sectional	Score ≥ 4 points based on Lüneburg criteria			CP: 67	CP: -	CP: 27 (40.29)	CP: 60[1] (-)	CP: 22.7 (15-37.9)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
Min *et al*[31], 2018	Prospective cohort	EUS criteria and/or	EUS criteria (unspecified number in each category)	Toxic/metabolic: 54 (59.3)	CP: 91	CP: -	CP: 34 (37.36)	CP: 48.6 (10.4)	CP: 26.1 (7.8)
		secretin stimulation testing		Idiopathic: 17 (18.6)	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -
				Hereditary: 13 (14.3)					
				Autoimmune: 5 (5.5)					
Gupta *et al*[33], 2019	Prospective cohort	Clinicoradiological and EUS			CP: 38	CP: White 35 (92), black 3 (8)	CP: 19 (50)	CP: 44 (10.7)	CP: 26.7 (5.9)
					Controls: -	Controls: -	Controls: -	Controls: -	Controls: -

Kanakis *et al*[7], 2020	Retrospective cohort	Clinicoradiological			CP: 239 Controls: -	CP: White 43 (88), minorities: 6 (12) Controls: -	CP: 37 (15.48) Controls: -	CP: 56[1] (-) Controls: -	CP: 23 (8) Controls: -
Hart *et al*[34], 2021	Cross sectional	Clinicoradiological	Cambridge classification (unspecified number in each category)	-	CP: 282 Controls: -	CP: White race (87.2), minorities (12.8) Controls: -	CP: 145 (51.41) Controls: -	CP: 56[1] (-) Controls: -	CP: - Controls: -
Vujasinovic *et al* [8], 2021	Retrospective cohort	2002 Asia-Pacifc consensus report		Alcohol and smoking: 40 (33.9) Smoking only: 12 (11) Alcohol only: 7 (5.9) Hereditary: 21 (11.8) Immunological: 23 (14.4) Efferent duct factors: 11 (9.3)	CP: 118 Controls: -	CP: - Controls: -	CP: 49 (41.52) Controls: -	CP: 53.1 (16.3) Controls: -	CP: 23.9 (4.4) Controls: -
Tang *et al*[35], 2021	Cross sectional	ICD-9 based codes	M-ANNHEIM clinical stage 0: 6 (5.8), I: 59 (56.7), II: 26 (25.0), III: 8 (7.7), IV: 5 (4.8)		CP: 104 Controls: -	CP: - Controls: -	CP: 31 (29.8) Controls: -	CP: 46.08 (14.43) Controls: -	CP: 21.43 (2.85) Controls: -

[1]Number of subjects who consented to Dual-energy X-ray Absorptiometry scan if detailed in the studies

CP: Chronic pancreatitis; BMI: Body mass index; EUS: Endoscopic ultrasound; ICD: International Classification of Diseases.

RESULTS

Literature search

The literature search yielded 2081 results and after the removal of 20 duplicates, 2061 citations underwent title and abstract screening. The exclusion of 1979 citations due to lack of relevance to the research question resulted in 82 citations for full-text review. The review of their full texts enabled exclusion of 61 citations and finally 21 studies were included[7,8,17-35] (Supplementary Table 2). The excluded studies had incorrect study design (n = 27), wrong outcomes (n = 17), ineligible patient population (n = 8), non-English language (n = 6), wrong setting (n = 3), and duplicate center or overlapping populations (n = 1) (Supplementary Table 3). The search and selection processes are summarized in Figure 1.

Study design

All the eligible studies were observational studies and included 14 cross-sectional and 7 cohort studies (described in Table 1). Among the cohort studies, four were performed prospectively[25,26,31,33], and remaining three had retrospective design[7,8,35]. Most of the studies originated from European

Table 2 Characteristics of study outcomes, *n* (%)

Ref.	Population	Outcome definition	Osteoporosis	Osteopenia	Pathologic fracture
Morán *et al*[17], 1997	CP: 14	T score < -2.5	CP: 3 (21.4)	CP: 10 (71.4)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Haaber *et al*[18], 2000	CP: 58	Z score < -2	CP: 13 (22.4)	CP: 36 (62)	CP: -
	Controls: -	Z score < -1.0	Controls: -	Controls: -	Controls: -
		Unavailable			
Dujsikova *et al*[19], 2008	CP: 73	T score < -2.5	CP: 4 (5.5)	CP: 19 (26)	CP: 1 (1.3)
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Undefined			
Tignor *et al*[20], 2010	CP: 3192	Not studied	CP: -	CP: -	CP: 154 (4.8)
	Controls: 1436699	Not studied	Controls: -	Controls: -	Controls: -
		Vertebral, hip, and wrist fractures using ICD-9 codes			
Sudeep *et al*[22], 2011	CP: 31	T score < -2.5	CP: 9 (29)	CP: -	CP: -
	Controls: 35	T score -1 to -2.5	Controls: 3 (8.5)	Controls: -	Controls: -
		Unavailable			
Joshi *et al*[21], 2011	CP: 72	Z score < -2	CP: 22 (30.5)	CP: -	CP: 0
	Controls: 100	Unavailable	Controls: -	Controls: -	Controls: -
		Undefined			
Duggan *et al*[23], 2012	CP: 62	T score < -2.5	CP: 18 (33)	CP: 21 (39.6)	CP: -
	Controls: 66	T score -1 to -2.5	Controls: 6 (10.1)	Controls: 20 (33.8)	Controls: -
		Unavailable			
Sikkens *et al*[25], 2013	CP: 40	T score < -2.5	CP: 4 (10)	CP: 18 (45)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Prabhakaran *et al*[28], 2014	CP: 103	T score < -2.5	CP: 25 (30.1)	CP: 38 (45.7)	CP: -
	Controls: -	T score -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Bang *et al*[26], 2014	CP: 11972	M80.0-M81.9 based on ICD-10 code	CP: 898 (7.5)	CP: -	CP: 1055 (8.8)
	Controls: 119720	Unavailable	Controls: 4070 (3.3)	Controls: -	Controls: 8485 (7)
		Spine, humerus, distal forearm, and proximal femur based on ICD-10 codes			
Duggan *et al*[27], 2015	CP: 29	T score < -2.5	CP: 9 (31)	CP: 13 (44.8)	CP: -
	Controls: 29	T score -1 to -2.5	Controls: 2 (6.8)	Controls: 15 (51.7)	Controls: -
		Unavailable			
Munigala *et al*[24], 2016	CP: 3257	Unspecified ICD-9 codes	CP: -	CP: -	CP: 153 (4.6)
	Controls: 450655	Unavailable	Controls: -	Controls: -	Controls: 9325 (2)
		ICD-9 codes: vertebral (805.2, 805.3, 805.4,805.5, 805.6, 805.7), hip (820.0, 820.1, 820.2, 820.3, 820.8, 820.9), or wrist fractures (814.0, 814.1, 813.4, 813.5)			

Kumar *et al*[29], 2017	CP: 102	Z score < -2	CP: 6 (5.8)	CP: 21 (20.5)	CP: -
	Controls: -	Unavailable	Controls: -	Controls: -	Controls: -
		Unavailable			
Stigliano *et al*[32], 2018	CP: 211	T score < -2.5	CP: 46 (21.8)	CP: 89 (42.1)	CP: 13 (6.1)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Occurring at the spine, hip and distal radius, and not associated with traumatic events			
Kuhlmann *et al* [30], 2018	CP: 67	T score < -2.5	CP: 18 (26.8)	CP: 34 (50.7)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Min *et al*[31], 2018	CP: 91	T score < -2.5	CP: 10 (22.2)	CP: 21 (46.6)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Gupta *et al*[33], 2019	CP: 38	T score < -2.5	CP: 21 (55.2)	CP: -	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		A fall from standing height or less that resulted in a fracture			
Kanakis *et al*[7], 2020	CP: 239	T score < -2.5 or history of fragility fracture	CP: 15 (30.6)	CP: 27 (55.1)	CP: 22 (9)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Hip or vertebral fracture not due to excess trauma			
Hart *et al*[34], 2021	CP: 282	T score < -2.5	CP: 48 (17)	CP: 110 (39)	CP: 6 (2.1)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Spontaneous fractures			
Vujasinovic *et al* [8], 2021	CP: 118	T score < -2.5	CP: 30 (25.4)	CP: 33 (27.9)	CP: 33 (27.9)
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Unavailable			
Tang *et al*[35], 2021	CP: 104	T score < -2.5	CP: 6 (5.7)	CP: 32 (30.7)	CP: -
	Controls: -	T score: -1 to -2.5	Controls: -	Controls: -	Controls: -
		Occurring in the hip, spine, or wrist			

CP: Chronic pancreatitis; ICD: International Classification of Diseases.

countries (n = 9), while the remaining were from the United States (n = 6), India (n = 4), China (n = 1), and Argentina (n = 1). Multicenter collaboration was noted in two eligible studies whereas the remaining 19 were performed at single centers. The controls of matched patients were also analyzed in seven studies which were from a healthcare system (n = 3)[20,22,24]; healthy community population (n = 3)[21,26,27]; or were not defined (n = 1)[28].

Population characteristics

The qualitative review of 21 studies included 20155 CP patients and 2007278 control patients (from seven controlled studies). The patients with CP were identified based on the combination of clinical, radiologic, endoscopic and/or histologic findings in 17 studies[7,8,17-19,21-23,25,27-34], and diagnosed through ICD-9/10 codes in four studies[20,24,26,35] (Table 1).

Prevalence of bone disease

We performed meta-analysis of 21 eligible studies which included 20155 CP patients evaluated for bone disease.

Prevalence of osteopenia in CP patients: In adult CP patients, the pooled prevalence of osteopenia was 41.2% (95%CI: 35.2%-47.3%, P < 0.0001; χ^2 = 63.0, df = 14, P < 0.0001; I^2 = 77.8%, τ^2 = 0.04)[7,8,17-19,23,25,27-32,34,35] (Figure 2A). All of the studies prevalence estimates had high heterogeneity since I^2 > 75%.

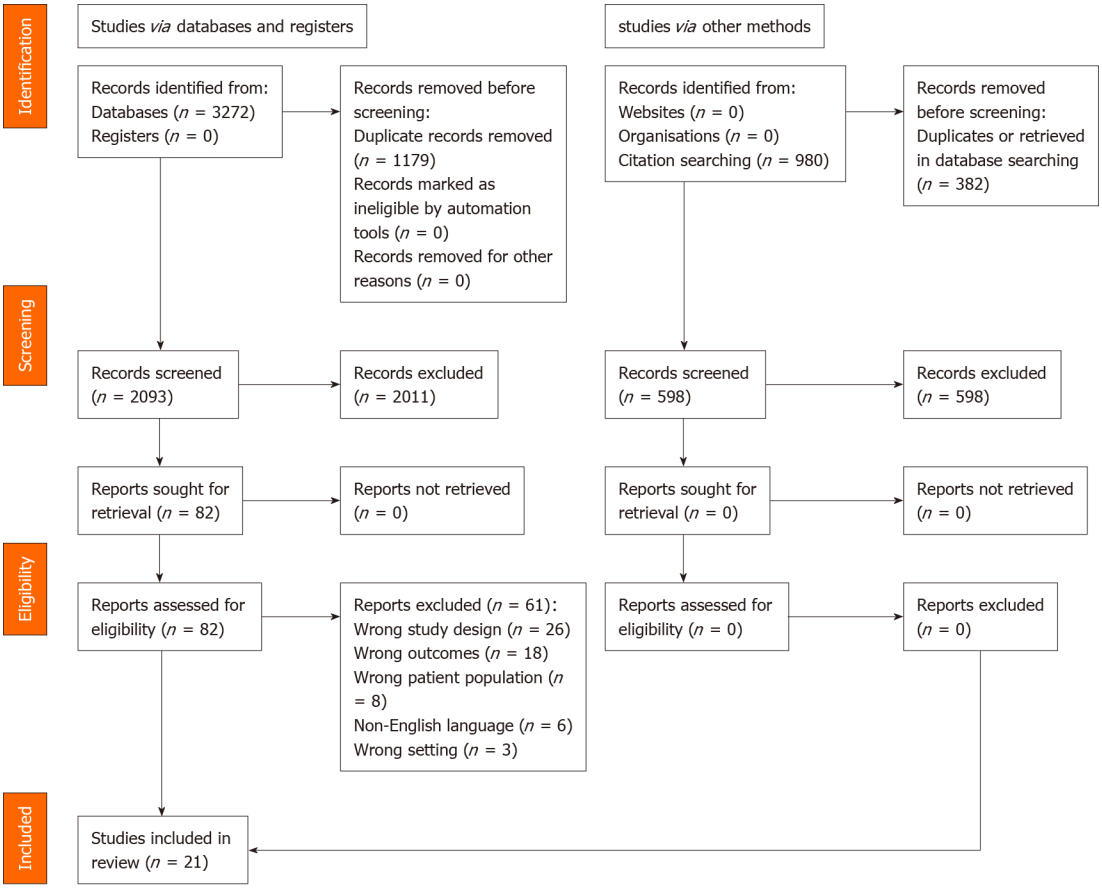


Figure 1 PRISMA flow diagram describing study selection.

Prevalence of osteoporosis among CP patients: The pooled prevalence of osteoporosis in CP patients was 20.9% (95%CI: 14.9%-27.6%; $P < 0.0001$; $\chi^2 = 290.33$, $df = 18$, $P < 0.0001$; $I^2 = 93.8\%$, $\tau^2 = 0.1$)[7,8,17-19,21-23,25-35] (Figure 2B). The pooled prevalence of osteoporosis in CP patients had high heterogeneity since $I^2 > 75\%$.

Prevalence of fragility fracture among CP patients: The pooled prevalence of fragility fracture was 5.9% (95%CI: 3.9%-8.4%, $P < 0.0001$; $\chi^2 = 186.0$, $df = 8$, $P < 0.0001$; $I^2 = 95.7\%$, $\tau^2 = 0.02$)[7,19-21,24,26,32,34,35] (Figure 2C). All of the studies prevalence estimates had high heterogeneity since $I^2 > 75\%$.

Factors impacting bone disease in CP patients: Various covariates underwent qualitative assessment for their association with CP related bone disease (Table 3).

Baseline patient characteristics: Female population was excluded in three studies[17,22,28] whereas 6376 (31.6%) female subjects were incorporated in the remaining 18 studies. In addition, three studies excluded post-menopausal females[19,25,33]. The race of CP patients was described in only five studies [7,20,24,33,34]. Its association with bone disease was investigated only by Hart and colleagues and they observed significantly higher burden among Caucasian population[34].

CP activity: The impact of CP severity on the bone outcomes was investigated in only one third of the eligible studies[17,19,27,28,31,32,34]. These studies observed no correlation with CP severity and alteration in bone mineral density. Moreover, the association of bone disease with duration of CP was also studied and lacked statistically significance[18,29,32,33,34,35]. The studies by Sikkens *et al*[25], and Kuhlman *et al*[30], observed a significant association of bone disease with exocrine insufficiency. Improved bone disease outcomes were observed in patients receiving PERT based on four large sized studies[8,26,30,34]. PERT usage and CP-related bone disease lacked statistical significance in five observational studies[17,22,23,28,32].

Inflammatory factors: Inflammation-based biomarkers such as C-reactive protein and interleukin (IL)-6 levels also yielded non-significant results in the investigation by two groups[27,32].

Endocrine factors: Among the eligible studies, hormones which regulate calcium metabolism have been widely studied. Serum 25-OH cholecalciferol had a positive correlation with bone density in three

Table 3 Characteristics of various covariates and their association with outcome measures

Ref.	PERT use, *n* (%)	Inflammatory markers: CRP/IL-6, mean ± SD	Vitamin D deficiency, *n* (%)	Serum PTH, mean ± SD	Alcohol exposure, *n* (%)	Smokers, *n* (%)	Diabetes, *n* (%)	Nutritional parameters	Relevant covariates findings
Morán *et al* [17], 1997	CP: 4 (28.57) Controls: -	CP: - Controls: -	CP: 7 (50) Controls: -	CP: - Controls: -	CP: 0 Controls: -	CP: - Controls: -	CP: - Controls: -	Mean serum albumin 3.8 g/dL, 4 (28.6) had BMI < 20. Non-significant associations between osteopathy and BMI	Non-significant associations between osteopathy and (1) CP severity (as per fecal fat or bicarbonate secretion assessments); (2) CP etiology; (3) Age; and (4) Vitamin D, PTH or calcium
Haaber *et al* [18], 2000	CP: 26 (44.82) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 40[1] (31) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -		Non-significant associations between osteopathy and (1) Duration of CP; and (2) Vitamin D and PTH
Dujsikova *et al* [19], 2008	CP: - Controls: -	CP: - Controls: -	CP: 63 (86.3) Controls: -	CP: - Controls: -	CP: 8 (10.95) Controls: -	CP: - Controls: -	CP: - Controls: -		Non-significant associations between osteopathy and severity of disease
Tignor *et al* [20], 2010	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -		No descriptions of regression analysis or covariate adjustment
Sudeep *et al* [22], 2011	CP: - Controls: -	CP: - Controls: -	CP: 16 (51.6) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	BMI correlated significantly with BMC (r = 0.426; P = 0.017). There was an inverse correlation between stool fat and BMC (r = -0.47; P = 0.03)	Non-significant associations between osteopathy and (1) EPI (as per 72-hour fecal fat); and (2) Vitamin D
Joshi *et al*[21], 2011	CP: 33 (45.83) Controls: -	CP: CRP < 0.32 (-) Controls: CRP < 0.32 (-)	CP: 62 (86.11) Controls: 85 (85) Significant association of Lumbar Z score with log vitamin D (beta: 0.274; P = 0.04)	CP: 43.38[1] (-) Controls: 84.87[1] (-)	CP: - Controls: -	CP: 7 (9.7) Controls: -	CP: 52 (72.2) Controls: -	Lumbar Z score was associated with BMI (beta: 0.276; P = 0.04), serum albumin was significantly lower in patients compared with controls [4.0 (0.6) *vs* 4.6 (0.7) g/dL, P < 0.001]	
Duggan *et al* [23], 2012	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 58 (93.5) Controls: 62 (93.9)	CP: 46 (74.19) Controls: 40 (60.6)	CP: - Controls: -	BMI < 20: low BMD: 15 (23.8) *vs* normal BMD 10 (1.1)	Higher T scores for the lowest age tertile (P = 0.003). Lower T-score for smokers (P = 0.002). Non-significant associations between T scores at any area and (1) CP severity; (2) EPI; and (3) Ssex
Sikkens *et al* [25], 2013	CP: 19 (47.5) Controls: -	CP: - Controls: -	CP: - Controls: -	CP: - Controls: -	CP: 1 (2.5) Controls: -	CP: 27 (67.5) Controls: -	CP: - Controls: -	A high BMI is predictive of a ''higher'' lowest T-score [Coeff: 0.58 (0.2); P = 0.003]	Significant association between osteopathy and untreated EPI (P = 0.013)
Prabhakaran	CP: -	CP: -	CP: 20 (19.41)	CP: 27.6	CP: 72 (69.9)	CP: -	CP: 39	-	Non-significant associations between osteopathy

et al[28], 2014				(39.8)			(37.86)		and (1) EPI (as per steatorrhea assessment); (2) CP severity; and (3) CP etiology
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Non-significant associations between osteopathy and vitamin D, PTH and alkaline phosphatase						
Bang *et al*[26], 2014	CP: 3545 (29.61)	CP: -	CP: -	CP: -	CP: 3651 (30.49)	CP: -	CP: -		Increased risk of fracture among smokers (HR, 1.8; 95%CI, 1.7-1.8) and alcohol related CP (HR, 2.0 *vs* 1.5; $P < 0.0001$)
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: 2753 (2.29)	Controls: -	Controls: -		
	Reduced fracture risk among PERT treated CP patients (HR, 0.8; 95%CI, 0.7-0.9)								
Duggan *et al*[27], 2015	CP: -	CP: CRP: 3.15 (-), IL-6: 5.61 (-)	CP: 20	CP: 47.1 (19.4)	CP: 27 (93.10)	CP: 23 (79.3)	CP: -	Lower T scores were associated with BMI ($P = 0.04$)	Lower T scores were associated with age ($P = 0.006$). Non-significant association with carboxy-terminal telopeptide of type I collagen; osteocalcin; Procollagen 1 amino-terminal propeptide
	Controls: -	Controls: CRP: 0.9 (-), IL-6: 3.58 (1.82)	Controls: 18	Controls: 46.3 (14)	Controls: 28 (96.55)	Controls: 10 (34.4)	Controls: -		
		Non-significant association with IL-6 and CRP	Lower T scores were associated with serum vitamin D ($P = 0.002$). No association with PTH						
Munigala *et al*[24], 2016	CP: -	CP: -	CP: -	CP: -	CP: 494 (15.16)	CP: 505 (15.5)	CP: -	A significant association of BMD in the columnar spine with vitamin D level (coefficient 0.13 g/cm^2; $P = 0.017$) and BMI (coefficient 0.14 g/cm^2 ; $P = 0.007$) were observed on univariate analysis	Increased fracture risk among males (adjusted OR, 1.73% (95%CI: 1.46%-2.05%); $P < 0.0001$), alcohol consumers (OR, 2.30), and smokers (OR, 1.97). Non-significant associations between osteopathy and age
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: 37146 (8.24)	Controls: 77926 (17.29)	Controls: -		
Kumar *et al*[29], 2017	CP: -	CP: -	CP: 69 (67.64)	CP: -	CP: -	CP: -	CP: 54 (52.94)	A MUST score (malnutrition score) of 1 or higher was associated with an increased risk for osteopenia and osteoporosis on Fisher's exact test ($P = 0.0037$)	Non-significant association between osteopathy and duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Non-significant association between osteopathy and vitamin D						
Stigliano *et al*[32], 2018	CP: 116 (54.97)	CP: -	CP: 119 (56.39)	CP: -	CP: 127 (60.18)	CP: 145 (68.72)	CP: 77 (36.49)	Observed significant association of BMI with osteopathy (OR 0.89;	Osteopathy more prevalent with increasing age (OR 1.06; $P = 0.0002$), female sex (OR: 3.44; $P =$

	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	95%CI: 0.83-0.96; P = 0.003)	0.0005). Non-significant association between osteopathy and (1) CP severity; (2) EPI (as assessed by fecal elastase); (3) Smoking; (4) Duration of CP; and (5) Alcohol exposure
	Non-significant association between osteopathy and PERT usage	Non-significant association between osteopathy and IL-6/CRP	Non-significant association between osteopathy, vitamin D and PTH						
Kuhlmann *et al*[30], 2018	CP: 28 (41.79)	CP: -	CP: -	CP: -	CP: 42 (62.68)	CP: 42 (62.68)	CP: 22 (32.83)	The underweight BMI category, had significant higher odds of osteopathy (OR: 7.40; 95%CI: 1.56-34.99; P < 0.001)	Lower Z scores associated with (1) EPI (P = 0.01); (2) Smoking (P = 0.02). Non-significant association with alcohol exposure
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
			Lower Z scores associated with vitamin D (P = < 0.001)						
Min *et al*[31], 2018	CP: -	CP: -	CP: -	CP: -	CP: -	CP: -	CP: -	Non-significant association with BMI	Non-significant association with (1) CP severity; (2) PERT usage
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Gupta *et al* [33], 2019	CP: -	CP: -	CP: -	CP: -	CP: 13 (34.21)	CP: 18 (47.36)	CP: 12 (31.57)		Low bone mass was associated with lower BMI. Non-significant association with CP duration
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Kanakis *et al* [7], 2020	CP: -	CP: -	CP: -	CP: -	CP: 130 (54.39)	CP: 132 (55.23)	CP: -	For patients, there was no association between total hip BMD and BMI (P = 0.753)	No descriptions of regression analysis or covariate adjustment
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
Hart *et al*[34], 2021	CP: 161 (57.09)	CP: -	CP: -	CP: -	CP: -	CP: 191 (67.7)	CP: 111 (39.36)	Higher osteopathy risk associated with low BMI (P ≤ 0.001)	Increased risk of osteopathy with white race (P = 0.017), age (P ≤ 0.001), female sex (P ≤ 0.01) and past or present smoking (P ≤ 0.01). No associations with (1) CP severity (per atrophy assessment); and (2) Duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
	Reduced osteopathy among PERT users (P = 0.02)								
Vujasinovic *et al*[8], 2021	CP: 104 (88.13)	CP: -	CP: -	CP: -	CP: 53 (44.91)	CP: 76 (64.4)	CP: 28 (23.72)		
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
	Reduced time to first fracture in PERT-treated patients								
Tang *et al*[35], 2021	CP: 51	CP: CRP: 0.75 (-), IL-6: 4.51 (-)	CP: 76 (73.07)	CP: 40.86 (-)	CP: 52 (50)	CP: 45 (43.26)	CP: 28 (26.92)	Independent predictors of osteopathy: BMI (OR, 0.72; 95%CI, 0.58-0.89; P = 0.003)	Non-significant association between osteopathy and (1) Age; and (2) Duration of CP
	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -	Controls: -		
		Non-significant	Non-significant						

	association between osteopathy and IL-6/CRP	association between osteopathy and PTH

[1]Number of subjects who consented to Dual-energy X-ray Absorptiometry scan if detailed in the studies.

CP: Chronic pancreatitis; EPI: Exocrine pancreatic insufficiency; PTH: Parathyroid; PERT: Pancreatic enzyme replacement; BMI: Body mass index; CRP: C-reactive protein; IL-6: Interleukin 6.

studies[21,27,30], whereas the remaining reported no significant associations[17,18,22,28,29,32]. Similarly, alkaline phosphatase and calcium had non-significant correlation with bone mineral density in two studies[17,28]. Serum PTH level did not correlate with bone disease in three studies[17,18,28], in contrast to the significant association found by Stigliano *et al*[32], Duggan *et al*[27], and Tang *et al*[35]. Novel bone turnover-based biomarkers *i.e.*, carboxy-terminal telopeptide of type I collagen, osteocalcin, procollagen 1 amino-terminal propeptide also had non-significant results in limited observational studies[27,35]. Thyroid-stimulating hormones and Insulin-like growth factors 1 were considered by Munigala *et al*[24], but no correlation was found with bone mineral density (BMD). Although hypogonadism was higher in subjects with low BMD in the study by Gupta e*t al*[33], no statistically significant difference was found. Exogenous hormone uses or replacement therapy was not investigated in any of the included studies.

Nutritional factors: The evidence linking BMI with bone disease outcomes has been conflicting. Whereas six studies described higher BMI as a protective factor[19,21-23,30,32,35], others reported an increased risk or non-significant findings[17,31,33,34]. Serum albumin was also explored in two studies with no mention of outcome association[17,21]. Min and colleagues studied the Malnutrition Universal Screening Tool, a validated nutritional assessment tool and observed that a score of 1 or more had significant association with osteopenia and osteoporosis ($P = 0.004$)[31]. Three studies described calcium supplement intake but did not study its relationship with bone disease[21,22,30].

Lifestyle factors: The definitions for exposure to alcohol and/or smoking were heterogeneous with limited evidence on their impact on bone outcomes[7,8,17,19,21,23-28,30,32-35]. Outdoor activity and sunlight exposure was only investigated by Joshi *et al*[21], and although correlated positively with vitamin D levels, their impact on osteoporosis was not studied.

Meta-regression

Meta-regression revealed significant association of PERT use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$] but not with osteopenia or fragility fractures. No associations with mean age, sex distribution, BMI, alcohol or smoking exposure, vitamin D deficiency, PTH levels, and diabetes with prevalence of osteopenia, osteoporosis or fragility fractures (Table 4).

Sensitivity analysis

Sensitivity analysis through forest plot calculation was performed using the "remove one study" method as described above. Confidence intervals and heterogeneity was measured through I^2 and no significant alterations were observed after removal of one particular study (Supplementary Table 4).

Table 4 Metaregression of outcome measures with various covariates

Covariates	Osteopenia		Osteoporosis		Fragility fracture	
	95%CI	*P* value	95%CI	*P* value	95%CI	*P* value
Sex distribution	0.2 (-1.2-1.5)	*P* = 0.8	0.5 (-1.9-2.9)	*P* = 0.7	0.3 (-3.2-3.7)	*P* = 0.9
Age	0.0 (0-1)	*P* = 0.6	0.0 (-0.1-0.0)	*P* = 0.3	0.0 (-0.5-0.4)	*P* = 0.7
DM	0.3 (-5.5-6.0)	*P* = 0.9	0.0 (-3.9-3.8)	*P* = 0.9	-13.8 (-107.4-79.8)	*P* = 0.3
Alcohol use	0.0 (-1.5-1.3)	*P* = 0.1	0.8 (-8.5-10.0)	*P* = 0.7	-0.5 (-5.4-4.4)	*P* = 0.3
Vitamin D	0.0 (-0.02-0.0012)	*P* = 0.4	-0.002 (-0.004-0.001)	*P* = 0.2	0.0 (-0.003-0.005)	*P* = 0.5
PTH levels	0.002 (-0.02-0.02)	*P* = 0.5	0.0 (-0.02-0.03)	*P* = 0.4	-	-
PERT	1.7 (0.6 -2.8)	*P* = 0.2	1.7 (0.6-2.8)	*P* < 0.0001	1.0 (-4.3-6.2)	*P* = 0.5
Smoking	0.0 (-3.6-3.6)	*P* = 0.9	-0.5 (-2.9-1.9)	*P* = 0.6	2.0 (-3.4-7.9)	*P* = 0.3
Mean BMI	NA	*P* = 0.9	0.0 (-0.12-0.21)	*P* = 0.6	0.5 (-17.2-18.2)	*P* = 0.8

DM: diabetes mellitus; PTH: Parathyroid; PERT: Pancreatic enzyme replacement; NA: *P* value calculated through Monte Carlo permutation, coefficient not available; -: Insufficient values.

Study quality and publication bias assessment

All the observational studies were assessed for quality in selection, comparability, and outcome/exposure domain and their scores ranged from 5-8 and were in good agreement (93% agreement, Cohen's κ = 0.87) (Supplementary Table 5). The comparability domain was variable based upon the presence or absence of controls. Visual inspection of funnel plot and Egger's regression for studies describing osteopenia ($P = 0.003$), and osteoporosis ($P < 0.001$) demonstrated significant publication bias (Figure 3).

DISCUSSION

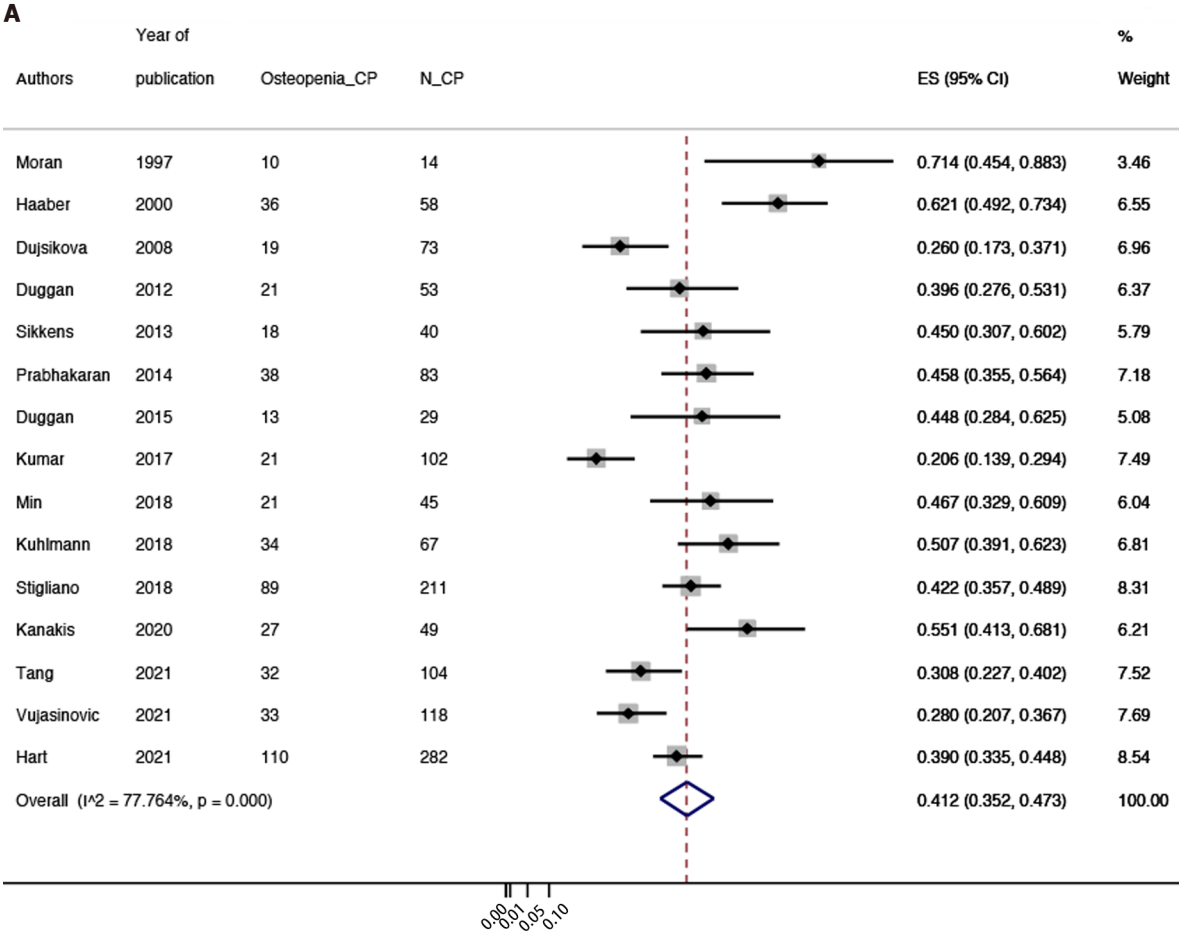
Through this systematic review we present the burden of osteopenia, osteoporosis, and fragility fractures in CP patients. The majority of the studies used bone mineral density to quantitate bone disease, which is a validated tool for use in the general population[36]. Our meta-analyses revealed osteopenia and osteoporosis to be prevalent in 41.2% and 20.9% of the CP population, respectively. We also observed a fracture risk of 5.9% among CP patients, which includes low trauma fractures predominantly in the hip, vertebrae and distal radius. These may cause pain, falls, and hospitalizations which may significantly impair the lives of CP patients along with pre-existing morbidities like malabsorption and chronic abdominal pain[37].

Although dedicated studies among CP patients are lacking, reduced bone mass and resultant fractures cause significant pain, reduced functionality, and quality of life in both men and women[38]. Chronic inflammatory conditions affecting the gastrointestinal tract, such as inflammatory bowel disease, celiac disease and chronic liver disease have been known to disrupt the balance between bone resorption and formation and cause bone disease. Histomorphometric analysis among alcohol related CP patients has revealed low cortical and trabecular bone thickness and endocortical apposition and growth rate as compared to controls[39]. Similarly, increased bone turnover and mineralization defects due to malnutrition have been observed in CP[27,40]. Although recent CP guidelines in the United States and Europe have suggested surveillance of bone-related disorders, studies report poor adherence to these guidelines, suggesting CP-related bone disease is an underappreciated clinical entity[7,8].

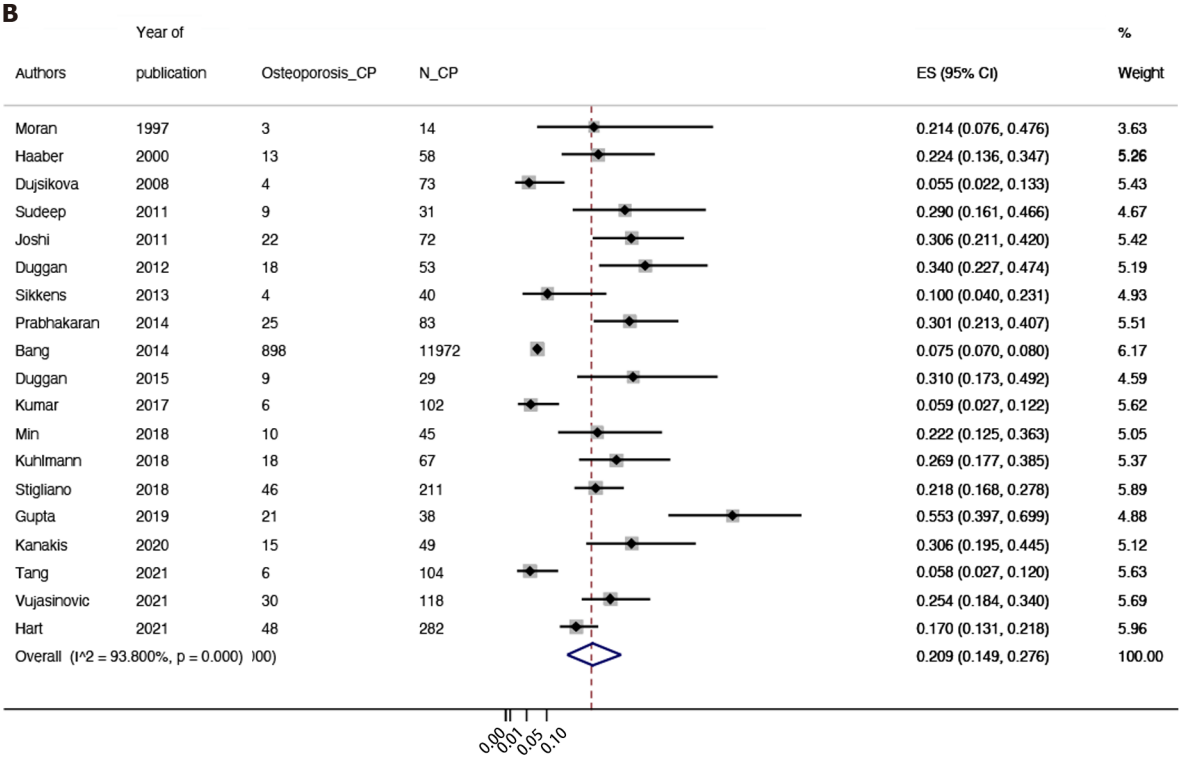
CP-related bone disease has been quantified previously by Duggan *et al*[9] in 2014 who observed similar prevalence of osteopenia and osteoporosis *i.e.*, 39.8% and 23.4%, respectively. Since this publication, over the past decade, several additional observational studies have investigated bone mineral density and metabolism in CP patients[7,8,24,25,27-31,33,34], and therefore, provide a unique opportunity to further investigate the evidence in this clinical domain. Hence, we appraised all the available literature, including the most recent publications, in an effort to contribute evidence for future by also uniquely quantifying fragility fractures which are significant complications of CP-related bone disease.

The current systematic review is powered by high-quality studies with large sample size and clearly defined study population and outcome measures. In addition, strengths of the study include a clearly articulated a priori analysis plan, a thorough search strategy and a conservative analysis. However, our study is limited by variability in the CP definitions from centers worldwide and spanning over 2

A


Authors
Year of publication
Osteopenia_CP
N_CP
ES (95% CI)
%
Weight
Moran 1997 10 14 0.714 (0.454, 0.883) 3.46
Haaber 2000 36 58 0.621 (0.492, 0.734) 6.55
Dujsikova 2008 19 73 0.260 (0.173, 0.371) 6.96
Duggan 2012 21 53 0.396 (0.276, 0.531) 6.37
Sikkens 2013 18 40 0.450 (0.307, 0.602) 5.79
Prabhakaran 2014 38 83 0.458 (0.355, 0.564) 7.18
Duggan 2015 13 29 0.448 (0.284, 0.625) 5.08
Kumar 2017 21 102 0.206 (0.139, 0.294) 7.49
Min 2018 21 45 0.467 (0.329, 0.609) 6.04
Kuhlmann 2018 34 67 0.507 (0.391, 0.623) 6.81
Stigliano 2018 89 211 0.422 (0.357, 0.489) 8.31
Kanakis 2020 27 49 0.551 (0.413, 0.681) 6.21
Tang 2021 32 104 0.308 (0.227, 0.402) 7.52
Vujasinovic 2021 33 118 0.280 (0.207, 0.367) 7.69
Hart 2021 110 282 0.390 (0.335, 0.448) 8.54
Overall (I^2 = 77.764%, p = 0.000) 0.412 (0.352, 0.473) 100.00
0.00 0.01 0.05 0.10

B


Authors
Year of publication
Osteoporosis_CP
N_CP
ES (95% CI)
%
Weight
Moran 1997 3 14 0.214 (0.076, 0.476) 3.63
Haaber 2000 13 58 0.224 (0.136, 0.347) 5.26
Dujsikova 2008 4 73 0.055 (0.022, 0.133) 5.43
Sudeep 2011 9 31 0.290 (0.161, 0.466) 4.67
Joshi 2011 22 72 0.306 (0.211, 0.420) 5.42
Duggan 2012 18 53 0.340 (0.227, 0.474) 5.19
Sikkens 2013 4 40 0.100 (0.040, 0.231) 4.93
Prabhakaran 2014 25 83 0.301 (0.213, 0.407) 5.51
Bang 2014 898 11972 0.075 (0.070, 0.080) 6.17
Duggan 2015 9 29 0.310 (0.173, 0.492) 4.59
Kumar 2017 6 102 0.059 (0.027, 0.122) 5.62
Min 2018 10 45 0.222 (0.125, 0.363) 5.05
Kuhlmann 2018 18 67 0.269 (0.177, 0.385) 5.37
Stigliano 2018 46 211 0.218 (0.168, 0.278) 5.89
Gupta 2019 21 38 0.553 (0.397, 0.699) 4.88
Kanakis 2020 15 49 0.306 (0.195, 0.445) 5.12
Tang 2021 6 104 0.058 (0.027, 0.120) 5.63
Vujasinovic 2021 30 118 0.254 (0.184, 0.340) 5.69
Hart 2021 48 282 0.170 (0.131, 0.218) 5.96
Overall (I^2 = 93.800%, p = 0.000) 0.209 (0.149, 0.276) 100.00
0.00 0.01 0.05 0.10

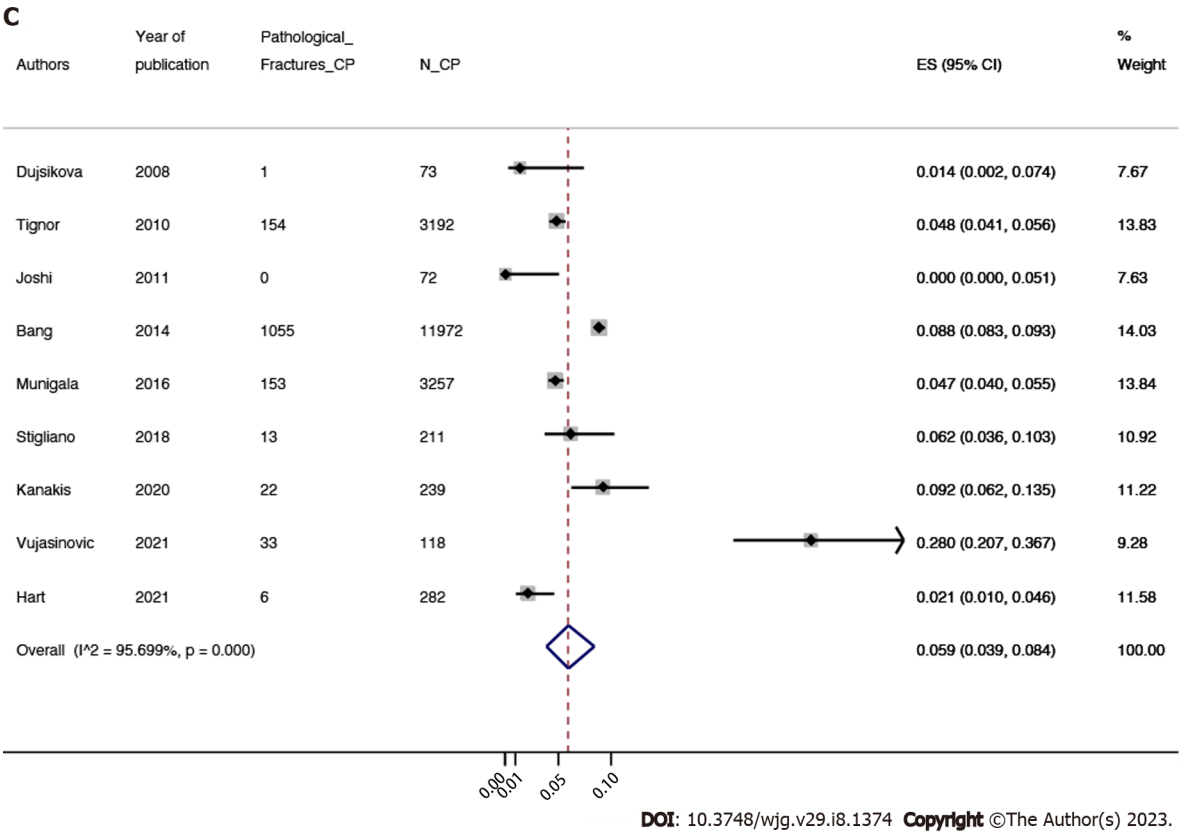
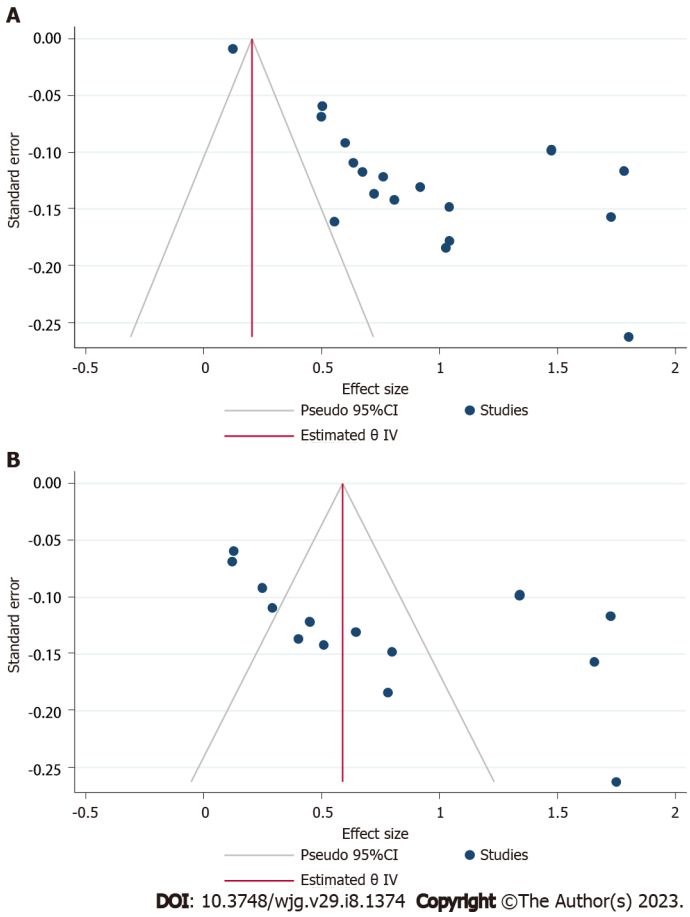


Figure 2 Forest plot demonstrating pooled prevalence of bone disease in chronic pancreatitis. A: Forest plot demonstrating pooled prevalence of osteopenia in chronic pancreatitis (CP); B: Forest plot demonstrating pooled prevalence of osteoporosis in CP; C: Forest plot demonstrating pooled prevalence of fragility fractures in CP. ES: Effect size; N: Total chronic pancreatitis population.

decades. Besides inconsistencies in definitions, high heterogeneity in the effect sizes is evident from I^2 mandated performance of metaregression and sensitivity analysis. The heterogeneity was not attributable to any specific study as demonstrated by sensitivity analysis. We observed significant association of osteoporosis with patients with PERT use on metaregression analysis. Although PERT use signifies severe disease, this association doesn't not establish causality and this in conjunction with limited information on the dosage of PERT or nutritional outcomes prevent substantial inferences to be drawn from this association[41]. We acknowledge significant publication bias, and a priori exclusion of studies in foreign language and conference abstracts[42]. These limitations prevent making categorical recommendations to patients.

Various mechanisms have been hypothesized to cause CP-mediated bone disease. Risk factors for CP such as cigarette smoking and alcohol exposure have been proven to alter the PTH-vitamin D axis and gonadal hormones and cause oxidative stress[39,43-46]. This clinical entity is also hypothesized to be driven by RANK ligand-induced osteoclastogenesis typically stimulated by inflammation-mediated nuclear factor-kappa B ligand[47]. Prior studies have also evaluated the relation of CP with inflammatory markers, such as IL-6, IL-1, and tumor necrosis factor-alpha[48]. Protein malnutrition lowers bone mass whereas deficiency of fat-soluble vitamins contributes to defects in mineralization and thus causes osteoporosis and resultant stress fracture[49,50]. CP is also characterized by low skeletal muscle, weight loss, and low mobility, all of which negatively impacts bone mass[51-53].

CP-related bone disease warrants further investigation to answer a few clinical queries. While CP patients are at risk, the impact of disease severity and duration on bone outcomes are unknown. Additionally, poor correlation between clinical symptomatology, severity and imaging findings in CP patients presents challenges for research[54]. We also systematically assessed various confounders including baseline clinical features and impact of bone turnover and inflammatory markers, albeit our results were unremarkable. The evidence on calcium supplements, hormone levels and outdoor activity among included studies was lacking. The studies that investigated mechanisms of systemic inflammation, bone turn over and malabsorption were underpowered, whereas those pertaining to vitamin D had conflicted evidence[17,18,21,22,27-30,32]. Among this at-risk group, efficacy of preventative therapy for osteoporotic fracture, drug interactions, and adverse effect also remain elusive.



DOI: 10.3748/wjg.v29.i8.1374

Figure 3 Funnel plots showing the publication bias assessment in studies describing patients with osteopenia and osteoporosis. A: Osteopenia ($Z = 2.9$, $P = 0.003$); B: Osteoporosis ($Z = 4.7$, $P < 0.001$).

CONCLUSION

In summary, this meta-analysis confirms significant bone disease in patients with CP. We observed significant association of PERT with CP related osteoporosis. Our study calls for improved methodology dedicated at delineation of confounders and studies targeting identification of at-risk CP patients, deeper understanding of mechanisms of CP related bone disease and their implications of treatment response. Fragility fractures are an important consequence of bone disease, which we have found to be increased in patients with CP. Screening strategies in this at-risk population with CP are needed as well as evaluating quality of life due to consequences of bone disease.

ARTICLE HIGHLIGHTS

Research background

Chronic pancreatitis (CP) is a multifactorial fibro-inflammatory syndrome is characterized by nutritional deficiencies, systemic inflammation, and etiological factors like alcohol or smoking may disrupt the balance between bone formation and resorption. These maladaptive alterations may cause osteopenia and osteoporosis, resulting in fragility fractures which result from low energy trauma and have significant implications on quality of life.

Research motivation

Multiple large-sized studies on CP patients including those from multicenter cohorts have investigated the burden of bone disease in CP as well as their association with nutritional, anthropometric, and inflammatory parameters. Thus, with quantitative data on covariates and fragility fractures available, a synthesis of evidence is pertinent.

Research objectives

This systematic review and meta-analysis sought to quantify the prevalence of osteopenia, osteoporosis,

and fragility fractures in CP patients and delineate clinical parameters which impact their occurrence.

Research methods

The study included systematic review and then metanalysis of studies describing bone disease in CP patients. A preregistered systematic search enabled identification of original studies from Cochrane Library, Embase, Google Scholar, Ovid Medline, PubMed, Scopus, and Web of Science, from inception until October 2022. The metanalysis was performed using random effect model and the outcomes of interest included prevalence of osteopenia, osteoporosis, and fragility fractures. To assess the association of these outcomes with covariates metaregression using random effect model was performed.

Research results

Twenty-one studies were included for systematic review and 18 studies were included for meta-analysis. The pooled prevalence of osteopenia and osteoporosis in CP patients was 41.2% (95%CI: 35.2%-47.3%) and 20.9% (95%CI: 14.9%-27.6%), respectively. The pooled prevalence of fragility fractures described among CP was 5.9% (95%CI: 3.9%-8.4%). Meta-regression showed no associations of bone outcomes in CP patients with mean age, sex distribution, body mass index, alcohol or smoking exposure, diabetes, serum parathyroid levels, and vitamin D deficiency with prevalence of osteopenia, osteoporosis or fragility fractures. A significant association of pancreatic enzyme replacement therapy use with prevalence of osteoporosis [coefficient: 1.7 (95%CI: 0.6-2.8); $P < 0.0001$]. Due to sparse data on systemic inflammation, CP severity, and bone mineralization parameters a formal meta-analysis was not feasible.

Research conclusions

This meta-analysis confirms significant bone disease: Osteopenia, osteoporosis, and fragility fractures in CP patients. Although pancreatic enzyme use had significant association with osteoporosis, the link between osteopathy and various patient or study-specific factors remains unclear. Further investigation is needed for delineation of at-risk population, to understand the mechanisms of CP-related bone disease, and assess the therapeutic response to treatment modalities.

Research perspectives

Our study calls for dedicated studies targeting delineation of confounders and identification of at-risk features in CP patients. There is also a knowledge gap in therapeutic response among CP patients with bone disease.

FOOTNOTES

Author contributions: Sheth SG and Chhoda A contributed to the study conception; Chhoda A, Addo NAA, and Hernandez-Woodbine MJ contributed to the study selection; Chhoda A and Hernandez-Woodbine MJ contributed to the data acquisition, analysis, and manuscript drafting; Grimshaw A contributed to the study accrual; Gunderson C contributed to the statistical supervision; Chhoda A and Nasir SA contributed to the study quality assessment; Ahmed A, Freedman SD, and Sheth SG contributed to the manuscript edition.

Conflict-of-interest statement: All the authors have no personal, financial or professional conflict of interest disclosures to declare.

PRISMA 2009 Checklist statement: The authors have read PRISMA checklist and revised according to PRISMA checklist.

Country/Territory of origin: United States

ORCID number: Ankit Chhoda 0000-0002-6232-0590; Maria Jose Hernandez-Woodbine 0000-0002-1481-617X; Alyssa Grimshaw 0000-0003-3985-0056; Steven D. Freedman 0000-0003-1255-9701; Sunil G. Sheth 0000-0003-0602-8509.

S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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