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EDITORIAL

Importance of *BRCA* mutation for the current treatment of pancreatic cancer beyond maintenance

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Abstract

In this editorial, we comment on pancreatic cancer (PC), one of the most aggressive and lethal cancers. Only minimal improvements in survival rates have been achieved over recent years. Available chemotherapeutic regimens have little impact, and surgical resection remains the only reliable curative approach. We address current treatment options for these patients, focusing on the usefulness of *breast cancer (BRCA)* gene mutation as a prognostic biomarker and predictor of response to chemotherapy. Superior survival outcomes have been reported in patients with PC and mutant *BRCA* gene treated with first-line platinum-based chemotherapy. Therefore, it appears appropriate to include *BRCA* gene status among clinical criteria used to select the chemotherapy regimen. In addition, maintenance treatment with poly(ADP-ribose) polymerase inhibitors has been found to improve progression-free survival in patients with PC and mutated *BRCA* whose disease does not progress after first-line platinum-based chemotherapy. This combination has therefore been proposed as the optimal treatment regimen for these patients.

Key Words: Pancreatic cancer; Treatment; *BRCA*; Mutation; Poly(ADP-ribose) polymerase inhibitor; Maintenance

Core Tip: Pancreatic cancer remains one of the most lethal malignant neoplasms, and available treatments have several limitations. Genetic studies are not currently recommended to support treatment selection. However, *breast cancer (BRCA)* gene

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mutation has been associated with superior survival outcomes in patients treated with platinum-based chemotherapy. Hence, it appears appropriate to consider the *BRCA* gene status of patients with this cancer among clinical criteria for the selection of first-line chemotherapy regimen.

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INTRODUCTION

Pancreatic cancer (PC) continues to be one of the most lethal malignant neoplasms, with a 5-year survival rate of only 5%[1]. It currently represents the third cause of cancer-related mortality and is expected to be the second by 2030[2]. Surgery is considered the sole potentially curative treatment; however, only 20% of patients diagnosed with PC are candidates for surgery at the time of diagnosis, and surgical resection is frequently followed by recurrence and therapeutic resistance[3].

There have been only limited advances in PC treatment over the past few decades. However, progress in basic research has recently generated increased molecular information on PC, improving knowledge of its biology and helping to explain the poor effectiveness of current therapies and the therapeutic resistance observed[4]. For instance, *KRAS*[5], *breast cancer* (*BRCA)*[6], and *ataxia-telangiectasia mutated*[7] genes play a major role in the prognosis and response to treatment of patients with advanced PC. Hence, the identification of patients with mutations in these genes can support the design of individualized therapies that may improve survival outcomes.

In this article, we evaluate the usefulness of *BRCA* gene mutation as a prognostic and predictive biomarker of the response to chemotherapy in PC patients, beyond their maintenance treatment.

CURRENT ADVANCED PC TREATMENT

Two first-line chemotherapy options are currently available for advanced PC, FOLFIRINOX and gemcitabine+nab-paclitaxel (GEM+Nab-P)[8]. These have both demonstrated superior overall survival (OS), progression-free survival (PFS), and response rates (RRs) compared to patients receiving monotherapy with gemcitabine. Specifically, the PRODIGE4/ACCORD11 study reported improved OS (11.1 *vs* 6.8 mo, respectively), PFS (6.4 *vs* 3.3 mo), and RR (31.6% *vs* 9.4%) in the FOLFIRINOX *vs* gemcitabine arm[9]. The MPACT study also reported improved OS (8.7 *vs* 6.6 mo), PFS (5.5 *vs* 3.7 mo), and RR (23% *vs* 7%) with GEM+Nab-P *vs* gemcitabine alone[10]. The higher percentage improvements obtained in the PRODIGE4/ACCORD11 study may be explained by the more favorable prognosis of the participants, who were less representative of the real-life clinical setting compared to those in the MPACT study. Specifically, the functional Eastern Cooperative Oncology Group score was 0 in 37% of PRODIGE4/ACCORD11 study participants *vs* 16% of MPACT study participants, the pancreatic head was tumor site in < 40% of the former *vs* 44% of the latter (60%-65% in clinical practice), the mean number of metastatic sites was two in the former *vs* three in the latter, the carbohydrate antigen 19-9 marker was elevated in 42% of the former *vs* 52% of the latter, and no patient over the age of 76 years participated in the former study. It should be noted that the higher survival and RRs in the PRODIGE4/ACCORD11 study were accompanied by a significant increase in hematologic and non-hematologic toxicity. This explains why FOLFIRINOX is frequently administered at a reduced dose or in modified form in the clinical setting. Finally, no randomized trials have been undertaken to compare these options, hampering evaluation of the optimal first-line treatment of PC. The only published studies have a retrospective or non-randomized prospective design, and the results have been contradictory[11-13]. Consequently, the choice of chemotherapy regimen largely depends on clinical variables, such as the performance status and previous comorbidities of patients[14,

15].

There are currently no recommendations for genetic studies to support the selection of PC treatments. One promising approach is the identification of mutations in genes involved in response mechanisms to DNA damage, such as *BRCA*, whose mutation has been associated with superior OS outcomes in PC patients treated with platinum-based chemotherapy[16]. This evidence is based on multiple *in vitro* studies and is supported by the longer OS observed in patients with advanced PC treated with platinum-based regimens who were *BRCA* mutation carriers than in those who were not (14 mo *vs* 5 mo; hazard ratio [HR] = 0.58; P = 0.08); however, this clinical trial was retrospective and only included 12 patients[17].

ROLE OF POLY(ADP-RIBOSE) POLYMERASE AND ITS IMPORTANCE IN MUTATED *BRCA*

Mutations in the genetic code must be detected and repaired to preserve genome integrity, avoiding the uncontrolled proliferation of healthy cells and possible development of cancer[18]. One DNA repair pathway detects single-strand DNA breaks. If defective, another pathway is involved in the detection of double-strand DNA breaks followed by their homologous recombination repair (HRR), using sister chromatids to restore the original DNA sequence in a high-fidelity mechanism[19]. Nuclear enzyme poly(ADP-ribose) polymerase (PARP) is responsible for detecting DNA damage and facilitating its repair. Specifically, PARP1, the main member of the PARP family, binds to and repairs both single- and double-strand DNA breaks[20]. Conversely, PARP1 inhibition results in persistent single-strand DNA breaks that lead to replication bifurcations and double-strand DNA breaks[21].

About 7% of PC patients possess *BRCA* mutations[22]. In these patients, the inhibition of PARP and resulting loss by the tumor of functional DNA repair pathways can synergically interact and produce the specific death of tumor cells. Studies in patients with ovarian, prostate, or breast cancer found that PARP inhibition enhances the activity of cytotoxic DNA agents including alkylating agents, topoisomerase inhibitors, and radiotherapeutic agents[23]. Hence, it appears plausible to assume distinct biological behaviors and responses to therapy in patients with advanced PC who have *BRCA* mutations, especially germline mutations. This has implications for the treatment selection and suggests that *BRCA* mutations may be a useful biomarker to predict the response to first-line treatment with platinum.

PLATINUM-BASED CHEMOTHERAPY IN PATIENTS WITH PC AND MUTATED *BRCA* GENE

Tumors with *BRCA* mutations are phenotypically characterized by their susceptibility to platinum-based chemotherapy, as noted above. *BRCA*-deficient cells accumulate double-strand DNA breaks, generating genomic instability and a greater predisposition to malignant transformation and progression. This is because the loss of HRR and PARP1 pathways leads to so-called synthetic lethality during DNA replication [24].

Patients with ovarian cancer who had mutated *BRCA*, either of somatic or germline origin, respond better to platinum-based chemotherapy regimens, with a superior prognosis and survival rate compared to those without this mutation[25]. In a study of 549 patients with metastatic PC, 78% of whom had at least one family member with a history of cancer, a median OS (mOS) of 8.1 mo (95% confidence interval [CI]: 7.5-9.0) was achieved by platinum-based chemotherapy, and 31% remained alive at 1 year. The mOS was higher in the patients with a family history of breast or ovarian cancer (8.5 mo; HR = 0.76; P = 0.042) and even higher in those with a family history of pancreatic and breast or ovarian cancer (14.8 mo; HR = 0. 43; P = 0.0003)[17]. According to these findings, a substantial subpopulation of patients with PC could benefit from platinum-based regimens. However, the underlying molecular mechanisms have not yet been elucidated, and further research is warranted in patients with *BRCA* mutant/deficient profiles. Other studies of PC patients receiving platinum-based chemotherapy have described a longer OS in those with a family history of breast, ovarian, or PC than in those with no family history of these cancers [26].

Cells with mutated *BRCA* are more susceptible to platinum and anthracyclines, which are selectively lethal in cells with HRR defects[27]. In a retrospective study of 36 PC patients treated with FOLFIRINOX, multivariate analyses confirmed a significantly longer mOS in patients with *vs* without homologous repair gene mutations (odds ratio [OR] = 1.47; 95%CI: 1.04-2.06; P = 0.04)[17]. In a study by Lowery *et al*[28] of 15 patients with advanced PC and germline *BRCA* mutation (*BRCA1* in 4 [27%] and *BRCA2* in 11 [73%]), 6 received platinum chemotherapy as first-line treatment, and 5 of these had a radiological partial response according to RECIST criteria, while the remaining patient had a complete response to the infusion of 5-fluorouracil, leucovorin, oxaliplatin, and irinotecan[28]. A study by Golan *et al*[26] of 71 patients with PC and a mutation in *BRCA1* (n = 21), *BRCA2* (n = 49), or both (n = 1) reported a longer mOS in patients treated with platinum than in those receiving other agents (22 *vs* 4.4 mo, respectively); the authors concluded that outcomes are more favorable in patients with PC who have *BRCA1* or *BRCA2* mutations than in those who do not[26].

USEFULNESS OF IPARP IN PC WITH MUTATED *BRCA*

In the highly influential POLO study[29], 154 patients with PC and germline *BRCA* mutation who showed no disease progression after at least 16 wk with FOLFIRINOX were randomly assigned to a group receiving maintenance therapy with olaparib, a PARP inhibitor (iPARP), or to a group receiving no maintenance treatment. Patients in the olaparib group showed a statistically significant improvement in PFS (7.4 mo *vs* 3.8 mo; HR = 0.53; 95%CI: 0.35-0.82), and 22% of them remained progression-free at 2 years compared to 9.6% in the untreated group, although there was no between-group difference in OS (18.9 *vs* 18.1 mo, respectively). Accordingly, the United States Food and Drug Administration approved olaparib as maintenance therapy for patients with advanced PC and germline *BRCA* mutation who show no disease progression after at least 16 wk of first-line treatment with platinum-based chemotherapy. In this regard, it has been reported that the iPARP-associated response does not depend on the germline or somatic origin of the *BRCA* mutation. Thus, one meta-analysis[22] describes eight studies that reported a response to PARPi in 24/43 (55.8%) patients with somatic *BRCA* mutation *vs* 69/157 (43.9%) patients with germline *BCRA* mutation, a non-significant difference (P = 0.399). In addition, five studies in the meta-analysis found no difference in PFS between patients with somatic *vs* germline *BCRA* mutations. The authors concluded that the response to iPARP therapy is similar between these types of patient.

Platinum-based chemotherapy has been combined with the administration of iPARP. An open-label, randomized, multicenter phase II trial was conducted on the efficacy of cisplatin plus gemcitabine with *vs* without veliparib in 50 patients with PC and germline-mutated *BRCA*. The RR was 74.1% for cisplatin plus gemcitabine with veliparib *vs* 65.2% for cisplatin plus gemcitabine alone (P = 0.55), obtaining a disease control rate of 100% with the former regimen *vs* 78.3% with the latter (P = 0.02). According to the authors, cisplatin plus gemcitabine is effective in advanced germline-mutated *BRCA* PC, and the addition of veliparib offers no improvement in therapeutic response[30-32]. These results support the selection of platinum-based chemotherapy as first-line treatment for patients with PC and germline *BRCA* mutation.

Various clinical trials are currently exploring the combination of iPARP with different chemotherapy and immunotherapy regimens (Table 1). It has been proposed that PARP inhibition induces tumor immunogenicity by increasing the tumor antigen load and the expression of programmed death-ligand 1 in tumor tissue, thereby increasing the susceptibility of patients with *BRCA* mutations to immunotherapy, as already demonstrated in breast cancer[33], small cell lung cancer[34], and ovarian cancer[35].

In summary, current studies suggest that *BRCA* mutation status may be a useful prognostic and predictive biomarker of the response to platinum in patients with PC, identifying those who may benefit from platinum-based chemotherapy as standard first-line treatment.

CLINICAL IMPLICATIONS

PC is associated with a poor prognosis and high resistance to chemotherapy. Few cytotoxic agents have demonstrated activity against this tumor, including platinum-based (FOLFIRINOX) and gemcitabine-based (GEM+Nab-P) regimens, and they

Table 1 Clinical trials on the combination of poly(ADP-ribose) polymerase inhibitor with chemotherapy and immunotherapy

Identifier	Phase	iPARP	Title	Status
NCT04548752	II	Olaparib	Randomized Phase II Clinical Trial of Olaparib + Pembrolizumab *vs* Olaparib Alone as Maintenance Therapy in Metastatic Pancreatic Cancer Patients with Germline BRCA1 or BRCA2 Mutations	Recruiting
NCT02890355	II	Veliparib	Randomized Phase II Study of 2nd Line FOLFIRI *vs* Modified FOLFIRI With PARP Inhibitor ABT-888 (Veliparib) (NSC-737664) in Metastatic Pancreatic Cancer	Active, not recruiting
NCT01585805	II	Veliparib	A Randomized Phase II Study of Gemcitabine, Cisplatin +/- Veliparib in Patients with Pancreas Adenocarcinoma and known BRCA/ PALB2 Mutation (Part I) and a Phase II Single Arm Study of Single-Agent Veliparib in Previously Treated Pancreas Adenocarcinoma (Part II)	Active, not recruiting
NCT01489865	I/II	ABT-888	A Phase I/II Study of ABT-888 in combination with 5-fluorouracil and Oxaliplatin (Modified FOLFOX-6) in Patients with Metastatic Pancreatic Cancer	Active, not recruiting
NCT03404960	I/II	Niraparib + Nivolumab Niraparib + Ipilimumab	PARPVAX: A Phase 1b/2, Open Label Study of Niraparib Plus either Ipilimumab or Nivolumab in Patients with Advanced Pancreatic Cancer whose disease has not progressed on Platinum-based Therapy	Recruiting
NCT03553004	II	Niraparib	Niraparib in Metastatic Pancreatic Cancer after previous Chemotherapy (NIRA-PANC)	Recruiting

iPARP: Poly(ADP-ribose) polymerase inhibitor.

deliver very modest benefits to the patient. There has been no comparative study of these agents to determine which is more appropriate as a first-line treatment, and this decision relies on the clinical characteristics and comorbidities of the patients. Two important issues must still be resolved: the best regimen for the personalization and optimization of first-line chemotherapy in patients with PC; and the ideal sequencing of chemotherapy lines, taking into account the accumulated toxicity and the molecular profile of the cancer.

As noted above, the *BRCA* gene encodes proteins essential for repairing double-strand DNA damage *via* the HRR pathway, and its mutation has been found to predict the response to first-line chemotherapy with platinum plus iPARP in patients with PC [26]. Thus, patients with advanced PC and germline *BRCA* mutation lived significantly longer when treated with platinum *vs* other cytotoxic agents[17]. In addition, maintenance treatment with iPARP has been found to improve the PFS of patients with PC and mutated *BRCA* whose disease does not progress after first-line platinum-based chemotherapy. Taken together, these findings support the selection of platinum-based regimens as first-line treatment of patients with PC and germline *BRCA* mutation[30-32].

Given the lack of evidence on the optimal treatment of patients with PC, it appears appropriate to consider the presence/absence of *BRCA* mutation among clinical criteria for the selection of first-line chemotherapy regimen.

CONCLUSION

An appreciable number of patients with PC have a mutated *BRCA* gene, and the ongoing development of drugs that target DNA repair pathways may offer relevant therapeutic benefits to this little-studied but clinically important sub-population. This defect in DNA repair pathways has the potential to improve outcomes in patients undergoing platinum-based chemotherapy, assisting individualized selection of the optimal first-line regimen.

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FRONTIER

Acetyl-CoA carboxylase inhibitors in non-alcoholic steatohepatitis: Is there a benefit?

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Abstract

De novo lipogenesis (DNL) plays an important role in the pathogenesis of hepatic steatosis and also appears to be implicated in hepatic inflammation and fibrosis. Accordingly, the inhibition of acetyl-CoA carboxylase, which catalyzes the rate-limiting step of DNL, might represent a useful approach in the management of patients with nonalcoholic fatty liver disease (NAFLD). Animal studies and preliminary data in patients with NAFLD consistently showed an improvement in steatosis with the use of these agents. However, effects on fibrosis were variable and an increase in plasma triglyceride levels was observed. Therefore, more long-term studies are needed to clarify the role of these agents in NAFLD and to determine their risk/benefit profile.

Key Words: Acetyl-CoA carboxylase inhibitors; Non-alcoholic steatohepatitis; Fibrosis; Steatosis; Firsocostat

Core Tip: Acetyl-CoA carboxylase inhibitors suppress de novo lipogenesis resulting in improvement in hepatic steatosis in both animal models and in patients with nonalcoholic fatty liver disease. However, the effects of these agents on hepatic fibrosis are inconsistent and they increase plasma triglyceride levels, casting doubt on their risk/benefit profile.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the commonest chronic liver disease in high-income countries, affecting 17%-46% of the general population[1]. NAFLD includes non-alcoholic fatty liver, characterized by isolated hepatic steatosis, and non-alcoholic steatohepatitis (NASH), where variable degrees of hepatic inflammation and fibrosis coexist with steatosis[2]. NASH is associated with increased risk for cirrhosis, hepatocellular cancer (HCC) and cardiovascular disease[3,4]. Diet and exercise, aiming at weight loss, is the cornerstone of management of NAFLD, but only a minority of patients achieves and maintains weight loss > 5%, which is essential for improvement in liver histology[2,5]. Several pharmacological agents have been evaluated in patients with NAFLD but none is currently licensed for use in this disease[2]. Therefore, there is an unmet need for safe and effective treatments in patients with NASH.

NON-ALCOHOLIC FATTY LIVER DISEASE

The pathogenesis of NASH is complex and multiple pathways, including insulin resistance, inflammation, oxidative stress and apoptosis are implicated[6]. De novo lipogenesis (DNL), defined as the synthesis of fatty acids from non-lipid sources, is pivotal in the development and progression of NASH. DNL is increased in patients with NAFLD and appears to be responsible for up to 38% of intrahepatic triglyceride content in this population[7]. In addition to its contribution to the development of hepatic steatosis, DNL also promotes fibrosis by activating hepatic stellate cells (HSC), which are the principal contributors to liver fibrosis[8,9]. Acetyl-CoA carboxylase (ACC) catalyzes the ATP-dependent carboxylation of acetyl-coenzyme A (CoA) to form malonyl-CoA, which is the rate-limiting and key regulatory step in DNL[10]. ACC exists as two isoenzymes that are encoded by two different genes; ACC1 is cytosolic whereas ACC2 is located at the mitochondrial membrane[10].

Given the central role of ACC in DNL and the implication of the latter in the pathogenesis of NAFLD, ACC might represent an attractive therapeutic target in this disease. Indeed, early studies showed that liver-specific, genetic inactivation of ACC protects against the development of hepatic steatosis[11,12]. More recently, several orally available, liver-specific, dual ACC1/ACC2 inhibitors have been developed and are being evaluated in the management of NAFLD (Table 1). Perhaps the most promising is firsocostat, formerly known as GS-0976. In mice with NASH, this agent improved hepatic steatosis and also reduced hepatic inflammation[13,14]. However, an increase in serum triglyceride, glucose and insulin levels as well in total body fat mass was observed[13,14]. In another study, a structural analog of GS-0976 reduced hepatic steatosis and hepatic insulin resistance in high-fructose-fed rats[15]. However, a 30%-130% increase in plasma triglyceride levels was again observed, which was attributed to an increase in very low density lipoprotein production and a decrease in triglyceride clearance by lipoprotein lipase[15]. Other ACC inhibitors also showed promise in ameliorating hepatic steatosis in rodent models of NASH. ND-630 reduced hepatic steatosis in Zucker diabetic fatty rats[16]. In addition, PF-05221304 not only improved liver steatosis in a rat model of NASH but also reduced hepatic inflammation[17].

In addition to the reduction in hepatic steatosis, ACC inhibition also appears to ameliorate hepatic fibrosis (Table 1), which is the strongest predictor of mortality in NASH[18-20]. In recent studies, firsocostat and a structural analog of this agent inhibited the activation of HSCs and reduced hepatic fibrosis both *in vitro* and in animal models of NASH[9,13,14]. PF-05221304 also prevented the activation of primary HSCs to myofibroblasts *in vitro* and reduced fibrosis in choline-deficient, high-fat-fed rats[17]. In contrast, MK-4074 did not affect fibrosis in a rat model of NASH, suggesting that the effect of ACC inhibition on fibrosis might be agent-specific [21]. On the other hand, another liver-specific, dual ACC1/ACC2 inhibitor, ND-654,

Table 1 Major findings of preclinical and clinical studies that evaluated the effects of acetyl-CoA carboxylase inhibitors in non-alcoholic steatohepatitis

Population	ACC inhibitor	Major findings	Ref.
Mice with NASH	Firsocostat (GS-0976)	↓ Hepatic steatosis, inflammation and fibrosis	[13, 14]
High-fructose-fed rats	A structural analog of firsocostat	↓ Hepatic steatosis; ↓ hepatic insulin resistance	[15]
Zucker diabetic fatty rats	ND-630	↓ Hepatic steatosis	[16]
Rat model of NASH	PF-05221304	↓ Hepatic steatosis, inflammation and fibrosis	[17]
Rat model of NASH	MK-4074	No effect on hepatic fibrosis	[21]
Rat model of NASH	ND-654	↓ Hepatic steatosis; Delayed progression of hepatocellular cancer	[22]
10 patients with NASH	Firsocostat	↓ Hepatic steatosis and fibrosis	[23]
126 patients with NASH	Firsocostat	↓ Hepatic steatosis and tissue inhibitor of metalloproteinase-1 levels	[24]
392 patients with NASH and bridging fibrosis or compensated cirrhosis (F3-F4)	Firsocostat	↓ Hepatic steatosis and stiffness	[25]
Healthy subjects	PF-05221304	Dose-dependent suppression of de novo lipogenesis	[26]
Overweight and/or obese adult males	ND-630	Suppression of de novo lipogenesis	[27]
30 patients with non-alcoholic fatty liver	MK-4074	↓ Hepatic steatosis	[28]

not only reduced hepatic steatosis but also delayed the progression of HCC in a rat model[22].

Preliminary studies suggest that ACC inhibition might also be effective in patients with NAFLD (Table 1). In a pilot, open-label, prospective study in 10 patients with NASH, administration of firsocostat for 12 wk reduced hepatic steatosis, assessed with magnetic resonance imaging (MRI), and fibrosis, assessed with both magnetic resonance elastography (MRE) and serum levels of tissue inhibitor of metalloproteinase 1 (TIMP-1)[23]. However, serum alanine aminotransferase levels did not change[23]. In a phase 2, randomized study in 126 patients with NASH, treatment with GS-0976 for 12 wk reduced hepatic steatosis, assessed with MRI, and TIMP-1 Levels more than placebo[24]. However, changes in MRE-measured liver stiffness did not differ among groups and an 11%-13% increase in serum triglyceride levels was observed in patients treated with GS-0976[24]. In a larger, phase 2b, randomized trial in 392 patients with NASH and bridging fibrosis or compensated cirrhosis (F3-F4), the incidence of the primary endpoint (a ≥ 1-stage improvement in fibrosis without worsening of NASH) did not differ between firsocostat and placebo[25]. However, firsocostat improved steatosis, increased the proportion of patients with ≥ 1-grade improvement in liver histology and improved liver stiffness evaluated by transient elastography and the Enhanced Liver Fibrosis Test compared with placebo[25]. Notably, serum glucose and insulin levels as well as body weight did not change in patients treated with firsocostat [25]. On the other hand, a mean increase in serum triglyceride levels by 42 mg/dL was observed in the firsocostat group[25].

Other ACC inhibitors also showed promising results in pilot clinical studies (Table 1). In healthy subjects, PF-05221304 dose-dependently suppressed DNL and was well-tolerated[26]. With doses yielding ≥ 90% DNL inhibition, asymptomatic increases in serum triglyceride levels and declines in platelet count occurred but these were not observed at ≤ 80% DNL inhibition[26]. A single dose of ND-630 was also shown to suppress DNL in overweight and/or obese but otherwise healthy adult males and was well tolerated[27]. Finally, in a randomized study in 30 patients with NAFL, treatment with MK-4074 for 4 wk decreased hepatic fat more than pioglitazone and placebo[28]. However, a 2-fold increase in plasma triglyceride levels was observed in patients treated with MK-4074 and not in the other groups[28]. It was shown that inhibition of ACC results in reduced intrahepatic content of polyunsaturated fatty acids, which in turn activates sterol regulatory element-binding protein-1c that increases hepatic production of very low density lipoprotein and therefore plasma triglyceride levels[28].

CONCLUSION

In conclusion, ACC inhibitors appear to represent a promising tool for ameliorating hepatic steatosis. The effect of these agents on hepatic fibrosis is less consistent and more studies are needed to assess their impact on NASH. In addition, given the high cardiovascular risk of patients with NASH, the increase in triglyceride levels during treatment with ACC inhibitors is a cause of concern and should be also be factored in the decision to administer them in this population.

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REVIEW

Therapeutic resistance in pancreatic ductal adenocarcinoma: Current challenges and future opportunities

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related deaths in the United States. Although chemotherapeutic regimens such as gemcitabine+ nab-paclitaxel and FOLFIRINOX (FOLinic acid, 5-Fluroruracil, IRINotecan, and Oxaliplatin) significantly improve patient survival, the prevalence of therapy resistance remains a major roadblock in the success of these agents. This review discusses the molecular mechanisms that play a crucial role in PDAC therapy resistance and how a better understanding of these mechanisms has shaped clinical trials for pancreatic cancer chemotherapy. Specifically, we have discussed the metabolic alterations and DNA repair mechanisms observed in PDAC and current approaches in targeting these mechanisms. Our discussion also includes the lessons learned following the failure of immunotherapy in PDAC and current approaches underway to improve tumor's immunological response.

Key Words: Pancreatic cancer; Metabolism; DNA repair; Therapy-resistance; Immunotherapy

Core Tip: With a five-year survival rate of 10%, pancreatic adenocarcinomas are one of the most aggressive forms of cancer. Despite extensive efforts, only a few drug combinations have been found to be effective in improving patient outcomes. The drug-resistant mechanisms active in pancreatic ductal adenocarcinoma contribute to the

quality classification
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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ineffectiveness of therapies. Through this review, we discuss key mechanisms that contribute to the development of resistant phenotype in pancreatic tumors and how these mechanisms are being sought as a target to treat this cancer.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor, with a 5-year overall survival of 10%. As the cause of approximately 47000 deaths annually, it is the third leading cause of cancer-related mortality in the United States and is expected to be the second primary cause of cancer-related deaths by 2030[1,2]. Surgical resection of the tumor remains the only curative option for patients with PDAC. However, due to late diagnosis, only a limited number of patients qualify for it. Relapse is common and often observed as early as two months post-surgery. Therefore, adjuvant chemotherapy is often prescribed to improve patient outcomes. For over a decade, gemcitabine was the mainstay for chemotherapy for resectable PDACs. The drug advanced the patient survival to 5.65 mo compared with 4.41 mo with 5-fluorouracil[3]. Recently, a combination therapy FOLFIRINOX (FOLinic acid, 5-Fluroruracil, IRINotecan, and Oxaliplatin) displayed better patient outcomes than gemcitabine[4]. The four-drug cocktail, although toxic, significantly improved survival in PDAC patients and is currently approved for both resectable and metastatic PDAC[5-9] (Table 1).

The complex pancreatic cancer biology is often attributed as the underlying cause of the poor chemotherapeutic response. This review will highlight the current knowledge of the therapeutic resistance mechanisms prevalent in PDAC and the opportunities PDAC tumor biology provides for its efficient targeting.

CURRENT THERAPIES IN PDAC

Gemcitabine

Gemcitabine has been a mainstay for PDAC treatment since 1997, when it was found to improve median and overall survival compared to 5-fluorouracil[3]. Gemcitabine (2′, 2′- difluorodeoxycytidine) is a difluoro analog of deoxycytidine which inhibits DNA synthesis through (1) inhibition of ribonucleotide reductase (RR), (2) inhibition of DNA polymerase (*via* diphosphate analog), or (3) mis-incorporation into the DNA, thus preventing chain elongation (*via* triphosphate analog)[10,11]. The inhibition of RR by the diphosphate analog depletes the deoxy-ribonucleotide pool essential for DNA synthesis.

Numerous mechanisms for gemcitabine inactivity have been demonstrated. Although resistance can be divided into innate and acquired forms, we will present evidence referring to both as "resistance" for this review.

The first interaction of gemcitabine with the cells occurs at the nucleotide transporter level. These transporters-concentrative nucleoside transporters (hCNTs) and equilibrative nucleoside transporters (hENTs) allow the transport of gemcitabine into the cells[12]. Evidence of the importance of nucleotide transporters for gemcitabine activity includes the observation that, in the absence of hENT1, PDAC patients treated with gemcitabine have reduced survival[13]. The enzyme deoxycytidine kinase (dCK) is the rate-limiting enzyme that converts gemcitabine into di-fluoro deoxycytidine mono-phosphate and is essential for gemcitabine-induced cytotoxicity[14]. Acquired resistant models demonstrate reduced expression of dCK in cells that do not respond to gemcitabine[14,15]. However, a recent analysis of the patient-derived xenograft PDAC model found no change in dCK levels in the gemcitabine-resistant tumors[16], indicating that mechanisms independent of dCK contribute to poor response to gemcitabine.

Table 1 Landmark trials for approved pancreatic ductal adenocarcinoma therapies

Treatment	Tumor characteristic	Primary endpoint	Ref.
Gemcitabine	Advanced PDAC	Median survival, 5.65 mo	Burris *et al*[3]
Gemcitabine + Erlotinib *vs* Gemcitabine	Locally Advanced or metastatic PDAC	Overall survival (OS), 6.24 mo *vs* 5.91 mo	Hoffmann *et al*[59]
FOLFIRINOX *vs* Gemcitabine	Metastatic PDAC	OS, 11.1 mo *vs* 6.8 mo	Conroy *et al*[4]
Gemcitabine + nab-paclitaxel *vs* Gemcitabine	Metastatic PDAC	OS, 8.5 mo *vs* 6.7 mo	Couvelard *et al*[60]
Gemcitabine + Capacitabine *vs* Gemcitabine	Resectable PDAC	OS, 28 mo *vs* 25.5 mo	Neoptolemos *et al*[8]

PDAC: Pancreatic ductal adenocarcinoma.

As mentioned earlier, when gemcitabine inhibits RR, the deoxy-ribonucleotide pool of the cells becomes depleted, leading to cell death. Overexpression of M1 and M2 isoforms, namely RRM1 and RRM2, is associated with reduced cellular response to gemcitabine[16-18]. Micro RNAs such as miR20a-5 and miR211 have been shown to downregulate RR, enhancing pancreatic cancer's sensitivity to gemcitabine and inhibiting cellular invasion[19,20]. Similarly, natural product, small molecule, and miRNA-based inhibition of RR sensitizes PDAC cells to gemcitabine[19-21-24]. Although strong *in vitro* data indicate RRM1/RRM2 play a key role in gemcitabine sensitivity, conflicting clinical outcomes have limited the utility of these enzymes for PDAC prognosis[25-28].

Other cellular processes such as epithelial-mesenchymal transition (EMT), mitogenic signaling, and tumor-stroma interaction also contribute to gemcitabine resistance [29]. Analysis of PDAC lines revealed that the EMT gene expression profile differs considerably between drug-sensitive and -resistant cells[30]. The drug-resistant cells showed reduced response to gemcitabine, 5-fluorouracil, and cisplatin, and expressed elevated levels of EMT marker Zeb1[30]. In addition, suppression of EMT enhanced the sensitivity of PDAC to gemcitabine by regulating the expression of nucleoside transporters[31].

5-Fluorouracil

Similar to gemcitabine, 5-fluorouracil belongs to the antimetabolite class of anti-cancer agents. 5-Fluorouracil inhibits the enzyme thymidylate synthetase (TS), which is responsible for methylation of deoxyuridine mono-phosphate to deoxythymidine mono-phosphate, a precursor for DNA synthesis. 5-Fluorouracil was the first drug to be approved as PDAC adjuvant therapy[32,33]. Although no longer used as monotherapy, 5-fluorouracil forms a part of the PDAC chemotherapeutic regimen FOLFIRINOX. Compared to gemcitabine therapy, combination therapy with FOLFIRINOX improved the overall survival and median progression-free survival of patients with metastatic PDAC[4]. Although any improvement in PDAC patient outcomes should be observed as a positive sign, the high toxicity of the drug regimen, limited patient eligibility for FOLFIRINOX, and prevalence of 5-fluorouracil resistant mechanisms may further limit the use this combination therapy in PDAC[34-38]. Multiple mechanisms have demonstrated to contribute to 5-fluorouracil resistance, such as alteration in (1) 5-fluorouracil metabolizing enzymes, (2) membrane transporters, and (3) pro-survival/ pro-apoptotic pathways. High TS expression is associated with poor survival in PDAC patients, however, the difference in survival is more significant in patients that received 5-fluorouracil based therapy[39,40]. The enzyme dihydropyrimidine dehydrogenase (DPD) catabolizes the 5-fluorouracil in the liver. In colorectal cancer patients receiving 5-fluorouracil based therapy, high DPD levels was associated with significantly shorter disease-free survival and overall survival[41]. In vitro analysis of PDAC cells lines and 5-fluorouracil-resistant sub-lines revealed that high expression of TS and DPDY is associated with poor 5-fluorouracil response[42].

Targeted therapies in PDAC

Comprehensive genetic analysis has revealed that pancreatic cancers are a host of numerous genetic mutations[43]. Mutation of *K-ras* is the most frequent genetic alteration observed in more than 90% of pancreatic cancer cases[44]. K-ras protein is a downstream signaling molecule activated by various transmembrane receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor, and c-met. EGFR, overexpressed in more than 40% of pancreatic

cancers, is associated with poor disease prognosis, invasion, and aggressive clinical behavior[45,46]. Given its importance, therapies targeting EGFR have been tested to determine their ability to improve the outcomes of PDAC patients. In one phase III trial, the addition of erlotinib (EGFR tyrosine kinase inhibitor) to gemcitabine-based therapy significantly improved the overall survival of PDAC patients[47]. A recent clinical trial compared the efficacy of gemcitabine + erlotinib in rash-positive pancreatic cancer patients and found similar one-year survival and better quality of life compared to patients on FOLFIRINOX[48]. Some trials however, have failed to show the clinical benefit of adding EGFR targeting drugs to PDAC chemotherapy[49-52]. Therapies targeting other molecular mechanisms active in pancreatic cancer have not shown beneficial effects, and EGFR targeting may have a place in PDAC therapy as precision medicine[53-57].

FUTURE OPPORTUNITIES TO TARGET PDAC

Pancreatic tumor metabolism

Pancreatic cancer is characterized by a dense stroma surrounding the tumor. This dense stromal region limits vascularization, creating an environment limiting oxygen and nutrient supply[58,59]. Limited oxygen gives rise to hypoxia that is associated with poor patient prognosis[59-61]. In an abundance of oxygen, the non-malignant cells produce most of their energy from mitochondrial oxidative phosphorylation (OXPHOS) while cancer cells exhibit an altered metabolism, first observed in the 1920s by Warburg[62], in which they produce most of their energy from glycolysis. Further, Warburg[62] observed that the majority of the glucose taken up by the cancer cells is converted to lactate rather than CO_2, an observation that has since been witnessed and verified by various researchers in various tumors, including PDAC[63-70]. Pancreatic cancer shows upregulation in glycolysis, pentose phosphate pathway (PPP), fatty acid synthesis, and purine/pyrimidine synthesis, and downregulation of enzymes involved in Kreb's cycle and the OXPHOS.

Analysis of the pancreatic cancer progression model revealed that the metabolic alterations precede tumor formation[71]. Metabolic rewiring in the early stages involves upregulated glycolytic and PPP. The altered metabolic profile allows quick ATP production and provides nucleotides and other metabolic intermediates required for proliferating cancer cells[72]. However, the suppression of OXPHOS can lead to excessive acid build-up within the cancer cells in the form of lactate. To circumvent this, pancreatic cancers express monocarboxylate transporters (MCT1 and MCT4) to efflux out lactate[73,74]. These metabolic adaptations, aided by the upregulation of glucose transporters GLUT1, allow the cancer cells to utilize glucose for their energy and biosynthetic needs. In addition, the molecular biology of pancreatic cancers, such as mutation of KRAS and P53, contribute to the so-called "glycolytic switch" in the PDACs by regulating genes like hexokinase-2, glucose transporters GLUT-1, and PKM2, and by promoting anabolic processes[75-78].

Altered tumor metabolism is also associated with poor therapy response in pancreatic tumors. Acquired gemcitabine-resistant models of pancreatic cancer show a marked increase in aerobic glycolysis that maintains the EMT phenotype and reduced responsiveness to the therapeutic agent[79]. The resistant cells exhibit elevated glycolytic enzymes HK2, LDHA and PKM2, and glucose transporter GLUT1. Below we discuss the central carbon metabolic pathways — namely, glycolysis, tricarboxylic acid (TCA) cycle, and the PPP — as therapeutic targets in pancreatic cancer.

Glycolysis as therapeutic target: Analysis of pancreatic tumors reveals that HK2 expression is upregulated in localized tumors as well as metastatic tumors compared to non-malignant tissues[80]. Since HK2 plays a crucial role in pancreatic tumors, efforts have been made to evaluate HK2 as a therapeutic target for pancreatic cancers. We were among the first to show that inhibition of glycolytic enzymes HK2 inhibits the growth and pro-survival signaling in pancreatic cancers[81]. In addition, inhibition of HK2 in pancreatic cancer cells suppresses their anchorage-independent growth and invasion[80]. The role of HK2 has also been implicated in gemcitabine resistance, as HK2 dimerization is enhanced in cells that do not respond to gemcitabine[82]. *In vitro* and *in vivo* analysis revealed that inhibition of HK2 enhanced the sensitivity of PDAC to gemcitabine. Similarly, in another study, inhibition of HK2 using chemical inhibitor 2-deoxyglucose enhanced resistant cells' sensitivity to gemcitabine[79].

PKM2: Pyruvate kinase (PK) is a glycolytic enzyme that catalyzes the conversion of phosphoenol pyruvate and ADP into pyruvate and ATP. Four isoforms of the enzyme

exist in vertebrates: PKR in erythrocytes; PKL in liver and kidney; PKM1 in adult muscle, brain, and heart; and PKM2 in most adult tissues and fetal tissues[83]. Phosphorylation of PKM2 at tyrosine residue 105 (Y105) is associated with reduced PKM2 activity and enhanced tumor growth[84,85]. Analyses of PKM isoform show abundance of isoform M2 in tumor cells compared to high levels of M1 in normal tissues[52,53]. In cancer cell lines, high PKM2 Levels are associated with proliferation, metastasis, and angiogenesis[54-56]. The role of PKM2 in pancreatic tumors is, however, controversial. Using the mice model of PDAC, a recent report demonstrated that although PKM2 expression is elevated in PDAC, the loss of PKM2 does not significantly affect the size of tumors or the survival of mice bearing PDAC[86]. Surgical specimens from 115 PDAC patients show that PKM2 expression is associated with better overall survival[87]. However, others have shown that high PKM2 expression correlates with poor patient outcomes[88,89]. Considering several observations demonstrating a vital role of PKM2 in pancreatic cancer survival, invasion, angiogenesis, metastasis, and drug resistance, we believe the PKM2 serves as an attractive target for the treatment of PDAC, even though its role in pancreatic cancer tumorigenesis is still unproven[90-95].

Lactate dehydrogenase (LDH): LDH is an enzyme that exists as a tetramer and catalyzes the conversion of pyruvate to lactate and *vice versa*. LDHA (LDH gene product) regulates pyruvate's conversion to lactate, thus preventing the entry of pyruvate into the TCA cycle. Deregulated expression of LDHA is observed in various tumors, including pancreatic, gastric, bladder, cholangiocarcinoma, lung, and endometrial cancers[96-102]. Numerous oncogenic signaling molecules, namely, HIF1 alpha, myc, FOXM1, and tyrosine kinase receptors, can regulate the level or the activity of LDH[96,103-106]. Elevated levels of LDH are associated with unfavorable prognoses for PDAC patient survival, chemotherapy response, and recurrence[107-112]. Preclinical studies have revealed that inhibition of LDH reduces the survival of PDAC cells[113,114].

PPP as therapeutic target: The PPP branches from glycolysis and contributes to the cancer phenotype through (1) synthesis of NADPH (oxidative PPP), which is important for redox regulation and fatty acid synthesis, and (2) supplying the proliferating cells with pentose sugar (non-oxidative PPP) for nucleic acid biosynthesis [115]. Accumulating evidence indicates that PPP plays a vital role in pancreatic tumor survival, metastasis, and therapy resistance. Our lab and others have shown that MYC regulates the activity of both oxidative and non-oxidative PPP through the regulation of G6PD and the RPIA (non-oxidative PPP) gene[78,116,117]. The regulation of RPIA *via* MYC appears to be under the directive of KRAS. The MAPK-MYC-RPIA-nucleotide biosynthesis pathway is shown to be important for KRAS-mediated maintenance of PDAC[78,116]. Considering that most PDAC patients (90%) express mutant KRAS, inhibition of PPP is an attractive strategy for developing more efficient pancreatic cancer therapies that would target KRAS-induced metabolic abnormalities. Our recent results found that pancreatic cancer cells resistant to erlotinib express elevated levels of G6PD. The upregulated G6PD prevents the induction of ROS in response to erlotinib, thus protecting the cells from drug-induced cytotoxicity[117]. The non-oxidative PPP has also been implicated in PDAC therapy resistance. Shukla *et al*[118] found that gemcitabine-resistant cells express enhanced carbon flux into the non-oxidative PPP, aided by elevated non-oxidative PPP enzyme levels. This alteration in metabolic flux allows elevated pyrimidine synthesis that contributes to gemcitabine resistance[118].

TCA cycle and OXPHOS as therapeutic target: Although cancer cells exhibit an elevated flux of glycolytic intermediate into branched pathways, the TCA cycle is still functional. The TCA cycle continues to provide proliferating cancer cells with energy, macromolecules and maintain the cellular redox balance. Recent reports have demonstrated the importance of the TCA cycle and OXPHOS in pancreatic cancer survival[119-123]. Due to their critical roles, the TCA cycle and OXPHOS have been tested as a therapeutic target for PDAC therapy. Three major approaches have been sought to this end: (1) Targeting TCA cycle enzyme/intermediates; (2) Targeting glutamine-dependent anaplerosis; and (3) Targeting the OXPHOS.

Glutamine, a non-essential amino acid, is considered an important energy source for PDAC along with glucose[124,125]. Accumulating evidence demonstrates that glutamine plays a vital role in PDAC proliferation, invasion, maintenance of redox balance, chemotherapy, and radiotherapy resistance, underlining glutamine metabolism as a potential therapeutic target[126-132]. However, conflicting results show that the presence of glutamine suppresses PDAC growth and invasion, dampening

enthusiasm for targeting glutamine metabolism[133-135]. A current clinical trial (NCT04634539) is analyzing whether adding glutamine improves efficacy and reduces the toxicity of PDAC chemotherapy. The results from this trial will shed light on the effect of glutamine on PDAC chemotherapy.

Two additional approaches, targeting the OXPHOS and the TCA cycle, have shown promise in preclinical evaluations, and agents targeting them are currently in clinical trials (Table 2). IACS-010759 inhibits mitochondrial complex one and has recently completed a phase I study in different tumor types, including advanced pancreatic cancers (Table 2). Although the preclinical data regarding the effect of IACS-010759 on pancreatic tumors is lacking, inhibition of OXPHOS complex one appears to be a promising strategy for overcoming drug resistance[136-139]. The anti-diabetic drug metformin has been tested and continues to be tested for its efficacy in PDAC (NCT01210911, NCT02336087, and NCT01666730). Although the experience with metformin in clinical settings has not resulted in improved patient outcomes, a recent meta-analysis indicated survival benefits in patients with PDAC and concurrent diabetes mellitus, highlighting a need for a personalized therapeutic approach for the success of this therapy[140-142].

CPI-613 or Demivistat (Table 2) is a TCA cycle targeting agent that inhibits the activity of pyruvate dehydrogenase and α- ketoglutarate dehydrogenase. In a phase 1 trial, 61% of patients achieved an objective response, and 3 (17%) patients achieved a complete response after receiving CPI-613[143].

Targeting PDAC DNA repair

Activating KRAS mutations are major drivers of malignant growth in PDAC and have remained undruggable until recent promising developments. Oncogenic KRAS-induced DNA replication stress drives genomic instability and tumorigenesis in PDAC. Genomic analysis have also revealed that modifications in "DNA damage control" is a prominent genetic alteration observed in PDAC[43]. Recently, genetic alterations in PDAC have been classified into four sub-types by Waddell *et al*[144]: (1) Stable; (2) Locally rearranged; (3) Scattered; and (4) Unstable. The "unstable" phenotype harbors mutations in the DNA damage repair (DDR), such as BRCA1, BRCA2, PALB2, and ATM. Mutations in ATM account for the most frequently occurring somatic mutations in approximately 4% of PDAC cases, followed by BRCA2, STK11, and BRCA1[144-147]. Given the important role these DDR genes play in a significant proportion of human PDACs, patients are likely to benefit from tailored, targeted therapies, including platinums, directed against specific DDR (Table 3). The following paragraphs will discuss these therapies.

Platinums: Platinum agents (cisplatin, oxaliplatin) cause DNA damage by forming platinum adducts on the DNA and causing DNA interstrand crosslinks[148]. Oxaliplatin is a component of the standard of care FOLFIRINOX, and platinum compounds alone are well suited in cancers that have a deficiency in the homologous repair (HR) pathway. Many studies have highlighted the advantageous use of platinum compounds for HR-deficient PDAC. Golan *et al*[149] showed a survival benefit (22 mo *vs* 9 mo) in platinum-treated *vs* platinum-naïve BRCA1/2 mutated advanced PDAC. Similarly, platinum improved overall survival in patients with HR-deficient PDACs and in patients with germline BRCA1, BRCA2, and PALB2 mutations[150,151]. Hence careful patient selection depending on the genetic make-up of the tumor would be essential for platinums to succeed.

Poly (ADP-ribose) glycohydrolase: Poly (ADP-ribose) glycohydrolase (PARG) is a macrodomain protein with exo- and endo-glycohydrolase activity[152,153]. It critically regulates DNA damage responses by removing poly (ADP-ribose) molecules (PARylation) on modified proteins during the DNA repair process. It is the primary PAR degrading enzyme and reverses poly (ADP ribose) polymerase (PARP) functions by hydrolyzing the ribose-ribose bonds present in PAR molecules. By preventing cytoplasmic PAR accumulation, PARG prevents PAR-mediated apoptosis, termed as parthanatos[154]. Inhibiting PARG causes DNA replication fork collapse, which leads to irreparable DNA damage and cell death. Recent studies have highlighted the benefits of selectively targeting PARG as an anti-cancer therapeutic strategy alone or in combination with other genotoxic therapies[155-157]. Targeting PARG was shown to enhance chemotherapeutic effects of DNA damaging agents, like oxaliplatin and 5-fluorouracil in PDAC, and was also synergistic with mitotic kinase, Wee-1 inhibition. In a siRNA screen with DNA replication factors, PARG inhibition was shown to be synergistic with TIMELESS, HUS1, MCM2, CHK1, and RFC2 proteins in an ovarian cancer model, indicating that combinations of PARGi and DNA replication stress

Table 2 Pancreatic ductal adenocarcinoma trials involving agents that target tumor metabolism

Drug	Target	Trial description	NCI trial number
IACS-010759	OXPHOS inhibitor	Phase I, in advanced cancers	NCT03291938
CPI-613	PDH/alpha KDH inhibitor	Phase I, combination with Gem + nab-paclitaxel	NCT03435289
CPI-613	PDH/alpha KDH inhibitor	Phase II, combination with FOLFIRINOX	NCT03699319
CPI-613	PDH/alpha KDH inhibitor	Phase III, combination with modified FOLFIRINOX	NCT03504423
Metformin and atorvastatin	Metabolic inhibitors	Metformin + Atorvastatin + Doxycycline + Mebendazole in cancers	NCT02201381
L-glutamine	Glutamine analog	Phase I, combination with Gem + nab-paclitaxel	NCT04634539

OXPHOS: Oxidative phosphorylation; PDH: Pyruvate dehydrogenase; KDH: Ketoglutarate dehydrogenase.

Table 3 Pancreatic ductal adenocarcinoma trials involving agents that target DNA repair

Drug	Target	Trial description	NCI trial number
M6620 (VX-970)	ATR	Phase I, M6620 and irinotecan hydrochloride in treating patients with solid tumors that are metastatic or cannot be removed by surgery	NCT02595931
AZD6738/olaparib	ATR/PARP	Phase II, Phase II trial of AZD6738 alone and in combination with olaparib	NCT03682289
BAY1895344	ATR	Phase I, testing the addition of an anti-cancer drug, BAY 1895344 ATR inhibitor, to the chemotherapy treatment (Gemcitabine) for advanced solid tumors, pancreatic cancer, and ovarian cancer	NCT04616534
Olaparib	PARP	Phase II, a study of pembrolizumab and olaparib for people with metastatic pancreatic ductal adenocarcinoma and homologous recombination deficiency or exceptional treatment response to platinum-based therapy	NCT04666740
Olaparib	PARP	Phase I, targeted PARP or MEK/ERK inhibition in patients with pancreatic cancer	NCT04005690
Olaparib	PARP	Phase II, a phase 2 study of cediranib in combination with olaparib in advanced solid tumors	NCT02498613
Olaparib	PARP	Phase II, olaparib in treating patients with stage IV pancreatic cancer	NCT02677038
Talazoparib	PARP	Phase II, measuring the effects of talazoparib in patients with advanced cancer and DNA repair variations	NCT04550494
Talazoparib	PARP	Phase I/II, a study of avelumab, binimetinib and talazoparib in patients with locally advanced or metastatic RAS-mutant solid tumors	NCT03637491
Niraparib	PARP	Phase II, niraparib in metastatic pancreatic cancer after previous chemotherapy (NIRA-PANC): A phase 2 trial	NCT03553004
Niraparib	PARP	Phase II, niraparib in patients with pancreatic cancer	NCT03601923
Rucaparib	PARP	Phase II, maintenance rucaparib in BRCA1, BRCA2 or PALB2 mutated pancreatic cancer that has not progressed on platinum-based therapy	NCT03140670
MK1775	WEE1	Phase I/II, a phase i and randomized phase II study of nab-paclitaxel/gemcitabine with or without AZD1775 for treatment of metastatic adenocarcinoma of the pancreas	NCT02194829

PARP: Poly (ADP ribose) polymerase.

inducers should be evaluated as potential therapeutic strategies for PDAC treatment [158]. A synthetic lethal relationship with PARG inhibition and DDR proteins like BRCA1, BRCA2, ABRAXAS, BARD1, and PALB2 was reported in an MCF7 breast cancer model[159]. Since genomic screens in PDAC have revealed alterations/mutations in similar DDR proteins, it is valuable to target PARG in such DDR-deficient PDAC tumors.

Wee-1: WEE1 kinase is an important cell cycle regulator of the G2-M checkpoint and is overexpressed in various cancers, including glioblastoma, breast cancer, osteosarcoma, and hepatocellular carcinoma[160-163]. It phosphorylates and inactivates CDK1 to allow for the repair of damaged DNA before entering mitosis. Wee-1 has regulatory roles in DNA replication stress and HR mechanisms[164-166]. In PDAC, Wee-1 expression is upregulated by a post-transcriptional mechanism regulated by RNA

binding protein, HuR[167], and its inhibition has been found to be effective in DNA repair-deficient PDAC cells[168]. In one study, Wee-1 inhibition was found to sensitize PDAC cells to gemcitabine chemo-radiation therapy[165]. Another study showed Wee-1 inhibition was synergistic with gemcitabine in p53-deficient PDAC xenografts[169]. Co-targeting WEE1 and ATM was shown to synergistically reduce cell proliferation and migration *via* downregulation of PDL-1 expression in pancreatic cancers[170]. Recently, it was also published that a combination of Wee-1 with another DNA repair target, PARG, enhances DNA damage and decreases cell survival in PDAC cells[171].

PARP: PARP is a DNA repair enzyme that plays a role in inflammation, regulation of cell death, transcription, and modulation of post-transcriptional gene expression. In response to DNA damage, PARP-1 could either promote cell survival and DNA repair or cause cell death when the damage is high[172]. PARP covalently adds Poly (ADP ribose) (PAR) chains onto its target proteins by consuming beta nicotinamide adenine dinucleotide (β NAD+). PAR further recruits other DNA repair proteins in the process of damage repair. Chemical competitive inhibitors of PARP enzymatic activity have gained interest as treatment options for many cancers, like ovarian, breast, uterine, and prostate[173], specifically for patients with tumors harboring somatic or germline defects/mutations in HR genes like *BRCA1/2*. Recent whole-genome sequencing studies done in patients with familial pancreatic cancer show that mutations in *BRCA2* gene accounts for 5%-10% of familial pancreatic cancers. In the Ashkenazi Jewish population with PDAC, this percentage increases to 13.7% and represents a major subgroup of PDAC cases that could benefit from PARP inhibitor (PARPi) therapy. In the context of synthetic lethality, impairment of two DNA repair pathways induces cell death and thus targeting HR deficient cells (*BRCA1/2* mutants or others) with PARP inhibitors was found to be lethal[174,175]. Following the success of POLO trial (Pancreas Cancer Olaparib Ongoing), in 2019 FDA approved olaparib (PARPi) as a maintenance therapy in patients with a germline BRCA mutated metastatic PDAC that had not progressed on first-line platinum therapy[174]. An increasing amount of ongoing preclinical and clinical studies suggest that PARPi in combination with either conventional chemotherapeutics (gemcitabine/nab-paclitaxel) or radiation therapy could benefit patients in the long run[176]. However, recent research suggests that although these respond greatly to PARP inhibitors, there is still 40%-70% of BRCA1/2-mutated cancers that fail to respond to PARPi therapy and in those settings PARPi cannot be used. Novel efforts to create a 'BRCAness-tumors harboring mutations in HR beyond BRCA1/2' phenotype in the cells by use of other small molecule inhibitors and their combination with PARPi is now being exploited. Bagnolini *et al*[174] discovered a small molecule disruptor of RAD51-BRCA2 interaction synergizes with olaparib in pancreatic cancer cells. Another study showed synthetic lethaility with PARPi therapy and FGFR1 blockade in pancreatic cancer[177]. Failure of PARPi therapy can also be attributed to acquired resistance mechanisms[178]. A study in pancreatic cancer showed a secondary mutation in BRCA2 emerged after the patient's exceptional response to platinum and PARPi therapy, which likely restored BRCA2 function in PARP inhibitor-resistant tumor cells[179]. Thus, careful evaluation and design of PARPi therapy should be pursued, and novel targets for PARPi beyond BRCA1/2 should be explored.

Other inhibitors of DDR pathway: Ataxia telangiectasia mutated (ATM) and RAD-3 related (ATR) are serine/threonine protein kinases that are involved in double/single-strand break repair and modulate DNA replication stress and DDR signaling[180-182]. ATM is one of the most commonly mutated DDR genes, and many whole genomic sequencing studies in PDAC have reported both somatic or germline ATM loss-of-function mutations. ATM loss drives pancreatic cancer progression, angiogenesis, epithelial-to-mesenchymal transition, and stemness[183]. Radiosensitization of cells with ATM loss/inhibition has been well documented in many tumor types, including pancreatic cancers[184-186]. ATM loss can also synergize with platinums and PARP inhibitor therapies, emphasizing its role in DNA repair. Specific to PDAC, two studies have shown that patients with ATM/ATR mutated tumors respond well to oxaliplatin-based chemotherapy, experiencing either improved progression-free survival or a stable disease[187,188]. Based on these data, multiple ongoing clinical trials (Phase I/II) involving ATM-deficient solid tumors have been initiated with DNA damage agents like PARP inhibitor therapies (olaparib, talazoparib, and niraparib), some of which accept pancreatic cancer patients. Chemical inhibition of ATM *via* small molecule inhibitors (AZD0156, AZD1390) is also being tested in combination with other agents in early stage clinical trials in patients with advanced solid tumors and brain tumors (NCT02588105, NCT03423628). Lack of ATM function may lead to

increased dependence on ATR for DDR, and thus ATR inhibition may be particularly potent in PDACs with somatic mutations in ATM. A recent study employing a multi-DDR interference strategy that included an ATR inhibitor and PARP and DNA-PKC inhibitor was shown to inhibit FOLFIRINOX-induced invasive clones in ATM-deficient PDAC tumors[189]. In 2012, a study tested VX-970, an ATR inhibitor, and found it sensitizes PDAC cells to radiation therapy *in vivo* and *in vitro*[190]. Another study found that a combination treatment of AZD6738 (ATR inhibitor) and gemcitabine induces PDAC regression by preventing checkpoint activation by gemcitabine[191]. The ATR inhibitors (VX-970, AZD6738, BAY18953[43]) are currently in the early stages of clinical development, like ATM inhibitors in patients with advanced solid tumors and lymphomas (NCT03188965, NCT03682289, NCT02595931, and NCT03718091), with or without other chemotherapeutic agents. Although these appear to be promising therapies, their clinical activity in PDAC patients is yet to be shown[183].

Immunotherapy

Immunotherapy has achieved promising outcomes in certain cancers, however is yet to be realized in PDAC[192-194]. Tumors with high tumor mutation burden (TMB, approximate mutations per megabase), such as melanomas and NSCLC, have shown to respond better to immunotherapy[195-197]. These TMBs are generally associated with mismatch repair (MMR) deficiency. PDACs intrinsically have low MMR deficiencies, which may explain the lower response to immunotherapy approaches such as immune checkpoint inhibitors (ICI)[198]. The immunosuppressive nature and "*T cell exhaustion*" further contributes to the poor response of PDAC to immunotherapy.

The PDAC is characterized by the presence of dense stroma in the tumor microenvironment. The stromal components include T cells (cytotoxic and regulatory) and myeloid cells such as tumor-associated macrophages (TAM). Infiltration with macrophages is observed in early PDAC tumor development stages and is associated with poor prognosis in PDAC patients[199-201]. These macrophages secrete immunosuppressive factors such as arginase and TGFβ, and thereby regulate T-cell mediated cytotoxicity and surveillance[200]. The myeloid-derived suppressor cells are immature myeloid cells that suppress T cell proliferation and promote ROS-induced T cell apoptosis[202,203]. The term "T cell exhaustion" is used for T cells' differentiation state in chronic antigen exposure. The exhaustion stage is driven by persistent T cell receptor signaling leading to ineffective T cell functioning[204-206]. Recent evidence has shown that the T cells present in the PDAC tumor microenvironment are defective in the production of interferon and tumor necrosis factors following peptide recognition[207,208]. However, the T cells with identical peptide specificity in the spleen retain functionality in tumor-bearing animals[209].

Some approaches that are currently under investigation for improving the immunological response of PDAC include as follow.

Cancer vaccines and immune checkpoint blockade: Monotherapies targeting programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) have not shown promising responses in PDAC. However, the therapy showed tumor regression and disease stabilization in other advanced cancers such as NSCLC, melanoma, and renal cancers[193]. Similarly, inhibition of PD-1 or PD-L1 failed to demonstrate a positive response in PDAC animal models[207,210-212]. Similar to ICI inhibitors, vaccine trials using vaccine-GVAX pancreas (granulocyte-macrophage colony-stimulating factor-secreting allogeneic pancreatic tumor cells) failed to improve overall survival in PDAC patients compared to single-agent chemotherapy[213]. Since the vaccines were able to recruit T cells, one approach to improve their efficacy would be to promote the activation of T cells, which may be achieved through the combination of vaccines with ICI[214]. Currently, clinical trials are underway for establishing the safety and efficacy of these GVAX with ICIs (NCT03153410, NCT02451982, and NCT02648282).

Targeting tumor associated macrophages: Another way to improve the efficacy of immunotherapies is to inhibit the immunosuppressive signaling that originates from the tumor microenvironment. For this, one strategy being tested is to inhibit myeloid cells. Researchers found that CD11b agonist reduces the total number of myeloid cells and improves survival in PDAC mice. In addition, when CD11b was combined with anti-PD-1, anti-CLTA-4, and gemcitabine, enhanced infiltration of tumor with CD8 T cells was observed[212]. Similarly, other studies have confirmed that targeting TAMs improves therapeutic and T-cell checkpoint immunotherapy response in PDAC models[215-217]. Blockade of Csf1/Csf1R (macrophage colony-stimulating factor

1/receptor) reduces collagen deposits and enhances CD8 T cell infiltration in the PDAC mice model[218]. Currently, a phase II trial is underway to determine the efficacy of cabralizumab (CSF1R inhibitor) in combination with nivolumab and chemotherapy in PDAC (NCT03336216).

Adoptive T cell therapy

Adoptive T cell therapy involves isolating T cells from tumors and then engineering, expanding, and infusing them back into the patients[219]. The chimeric antigen receptor (CAR) T cell therapy is an example of adoptive T cell therapy wherein the T cells are manipulated to express CAR to assist tumor recognition[220]. Antigen targets that are being tested for PDAC include mesothelin, prostate stem cell antigen, CEA, MUC1, and HER2[221]. However, the immunosuppressive microenvironment remains a hindrance in CAR-T cell therapy's success in PDAC[222,223]. Other barrier to the success of adoptive T cell therapy in PDAC include antigen selection and toxicities [224-226]. Still, a few promising outcomes have sustained hope for the use of this approach in PDAC. A phase 1 trial found that treatment of PDAC patients with mesothelin-targeting-CART-T cells stabilized disease in 2 out of 6 patients[227]. Similarly, analysis of efficacy and safety of MUC1-targeting CART-T cells found the therapy to be safe and successfully elevated the levels of CD4+ and CD8+ T cells at the tumor[228]. Currently, clinical trials are underway to determine MUC-1-targeted CAR-T cell therapy's efficacy and safety in patients with solid tumors, including PDAC (NCT02587689 and NCT02617134).

CONCLUSION

The PDAC remains an intractable disease that is slated to be the second leading cause of cancer-related deaths by 2030. Although surgical resection remains the only curative treatment option, late diagnosis, in addition to the patient's performance status, limits the scope of surgical intervention. Chemotherapeutic regimens such as gemcitabine+nab-paclitaxel and FOLFIRINOX has shown promise in improving patient survival; however, drug resistance remains a continuing challenge that has limited their efficacy. Two approaches that may improve PDAC patient outcomes include inhibiting the mechanism(s) that promote therapy resistance and targeting the key pathways essential for PDAC survival. The altered metabolism provides the PDAC cells with energy (ATP) and macromolecules essential for tumor growth. Additionally, studies have shown that metabolism plays a key role in PDAC therapy resistance. Similarly, PARP targeting therapies' success has once again brought the importance of DNA repair mechanisms in PDAC into the center. The limited success of immunotherapy has dampened the enthusiasm for targeting PDAC using this approach. However, the uncovering of mechanisms contributing to poor PDAC's response to immunotherapy has provided opportunities to test newer approaches. Even though the strategies mentioned above have shown promising pre-clinical results individually, a regimen targeting multiple aspects of PDAC will likely deliver a better clinical outcome in this deadly disease.

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REVIEW

Evaluation of botanicals as potential COVID-19 symptoms terminator

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Abstract

Information about the coronavirus disease 2019 (COVID-19) pandemic is still evolving since its appearance in December 2019 and has affected the whole world. Particularly, a search for an effective and safe treatment for COVID-19 continues. Botanical mixtures contain secondary metabolites (such as flavonoids, phenolics, alkaloids, essential oils *etc.*) with many therapeutic effects. In this study, the use of herbal treatments against COVID-19 was evaluated. Medical synthetic drugs focus mainly on respiratory symptoms, however herbal therapy with plant extracts may be useful to relieve overall symptoms of COVID-19 due to the variety of bioactive ingredients. Since COVID-19 is a virus that affects the respiratory tract, the antiviral effects of botanicals/plants against respiratory viruses have been examined through clinical studies. Data about COVID-19 patients revealed that the virus not only affects the respiratory system but different organs including the gastrointestinal (GI) system. As GI symptoms seriously affect quality of life, herbal options that might eliminate these problems were also evaluated. Finally, computer modeling studies of plants and their active compounds on COVID-19 were included. In summary, herbal therapies were identified as potential options for both antiviral effects and control of COVID-19 symptoms. Further data will be needed to enlighten all aspects of COVID-19 pathogenesis, before determining the effects of plants on severe acute respiratory syndrome coronavirus 2.

Key Words: COVID-19; Herbal therapies; Plant; SARS-CoV-2; Antiviral; Symptom

Core Tip: To stop the coronavirus disease 2019 (COVID-19) pandemic, extensive search is ongoing to develop effective and safe drugs against severe acute respiratory syndrome coronavirus 2. COVID-19 in a major way affects the respiratory system, but many patients also have gastrointestinal (GI) symptoms. Plants have beneficial effects

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Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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on various systems with their varied array of metabolites. In our study, the potential effects of herbal treatments against COVID-19 were examined. Their antiviral effects, their effects on the respiratory system, GI system, and other COVID-19 symptoms were investigated.

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INTRODUCTION

New coronavirus disease 2019 (COVID-19), which emerged in Wuhan in December 2019, spread rapidly and affected the whole world. The emergence, epidemiology, origin and evolution of COVID-19 has been extensively studied by Sun *et al*[1].

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been shown to carry out viral replication in the human host mainly through three main proteins and enzymes: 3-chymotrypsin-like protease (3CLpro), angiotensin-converting enzyme-2 (ACE2) and spike protein (TMPRSS2)[2,3]. ACE2 receptors are found in the body not only in the lungs but also in tissues such as the endothelium, heart, kidney and intestine[2]. This distribution makes many organs a target of COVID-19. The significance of ACE2, which is found in intestinal tissues, especially for amino acid uptake from foods, has been emphasized and it has been suggested that the intestine may be an important entry site for SARS-CoV-2[2-4] Azithromycin, chloroquine, lopinavir, remdesivir, ritonavir are options used in treatment and whose effects are evaluated[5]. Effective and safe drugs and vaccines are sought all over the world to prevent novel coronavirus. Minerals, herbs, herbal products, probiotics and vitamins are the main natural resources, whose effectiveness and also the usability of herbal medicines in COVID-19 were investigated and benefit, risk assessments were evaluated[6-8]. Truly, since the beginning of the COVID-19, herbal medicines have been used in China. A study has shown that 90% of the 214 patients were treated with the traditional herbal medicine, moreover, it is reported that some of them prevented COVID-19 infection in healthy individuals and enhanced the health state of patients with mild or severe symptoms[9,10]. Health scientists from the Zhongnan Hospital of Wuhan University included the use of traditional medicines in the guidelines for the treatment and prevention of COVID-19. The experts recommended using medicinal plants for the prevention of COVID-19, additionally, the use of different herbal mixtures were recommended according to the disease-stage[11].

Herbs and herbal products provide generous sources of primary and mostly secondary metabolites, which are valuable compounds (phenolics, flavonoids, tannins, alkaloids, essential oils, *etc.*) for prophylactic and chronical therapeutic purposes. Some of these metabolites in herbs and herbal mixtures have high chemical variety than the synthetics in stopping viral proliferations, and having antiviral activities[12]. Thus, botanicals can both show antiviral effects and relieve the symptoms of COVID-19 thanks to the different substance groups, which demonstrate different biological effects that will not be possible to achieve with a single synthetic drug. Based on this understanding, in this review, we offer all the potential interventions for COVID-19 infection according to previous and recently found antiviral effects of herbals. Considering the major transmission routes of COVID-19, where mostly ACE2 receptors found and the symptoms, the plants have been handled especially with their effects on the mostly respiratory and also gastrointestinal (GI) systems. Although ACE2, is typically expressed in epithelial cells of the airways, various GI symptoms in COVID-19 might be explained by the high expression of ACE2 in the digestive tract. Additionally, liver tests abnormalities, active viral replication in GI tract and patients' manifestations with GI symptoms (abdominal pain, diarrhea, nausea, vomiting) and possible fecal-oral transmission reveal the GI involvement in COVID-19[13].

Recent findings demonstrated that early blocking of COVID-19 with ACE2 inhibitors was one of the mechanisms used by novel drugs[14], on the other hand diabetes mellitus and hypertension enhanced the risk of COVID-19 infection, in spite of using ACE2 inhibitors[15-17]. Furthermore, unpredicted ACE2 upregulation by

ACE2 inhibitors, ibuprofen and angiotensin II type-I receptor blockers lead to need of identifying/using alternative ACE2 blockers[18]. Consequently, botanicals or natural products might be alternatively and selectively might block the ACE2 receptors without inhibiting the enzyme activity in order to treat and/or prevent COVID-19 spread in humans without increasing ACE2 expression in patients and therefore increased risk for COVID-19[19].

Clinical human studies showing the effect of plants on respiratory infections are presented as a table. Based on the pharmacological properties of plants, their practicality on COVID-19 symptoms have been evaluated. In the last part of the article, plants that inhibit ACE receptors, the research studies and their active compounds on COVID-19 also included and it is aimed to examine the plants from a broad perspective.

ANTIVIRAL EFFECTS OF HERBAL THERAPIES

Most of the respiratory diseases (approximately 80%) are caused by viral agents[20]. Viral respiratory diseases are responsible for high mortality and morbidity, especially in disadvantaged and sensitive elderlies and immunocompromised individuals[21, 22]. The main respiratory viruses are adenovirus, coronavirus, influenza virus, respiratory syncytial virus and rhinovirus[20]. Plants with antiviral effects and studies showing the effects of these on respiratory viruses are given in Table 1. Human clinical studies showing the effects of plants on respiratory tract infections are presented in Table 2.

EFFECTS OF HERBAL TREATMENT ON COVID-19 SYMPTOMS

Cough and fever are common symptoms in patients with COVID-19, including fatigue, shortness of breath, headache, muscle pain, sore throat, sputum, hemoptysis, diarrhea, dyspnea, rhinorrhea, chest pain, nausea, and vomiting[23]. COVID-19 symptoms in children are similar to those in adults and are relatively mild[24].

Although, the current synthetic drugs focus on mainly respiratory symptoms, herbal therapy can be used to relieve overall symptoms of COVID-19 with their bioactive ingredients[25]. The meta-analysis study, which included randomized controlled trial studies, found significant effects of the combination of western medicine and herbal therapies. Combined treatment has been effective in cough, fever, dry and sore throat, fatigue and overall GI symptoms. The combined therapy significantly improved the disappearance rate of cough and sputum production[26]. In another meta-analysis, it was found that the addition of Chinese herbal medicine for standard care improved the symptoms and signs of COVID-19 as well as decreased levels of C-reactive protein[27]. The effects of plants that can alleviate the symptoms of COVID-19 are summarized in Table 3. In addition, plants regarded as ACE inhibitors are shown in Table 4.

THE EFFECTS OF HERBS AND THEIR ACTIVE COMPOUNDS ON COVID-19

In recent years, artificial intelligence has often been used to discover natural products as medicine[28,29]. After the outbreak of COVID-19, computer models were used to investigate the effect of many plants and their components on SARS-CoV-2. Khaerunnisa *et al*[30], determined the COVID-19 Main Protease (Mpro) inhibitor effects of medicinal plant components in a molecular docking study. They suggested apigenin-7-glucoside, curcumin, catechin, demethoxycurcumin, epicatechin-gallate, luteolin-7-glucoside, and oleuropein, as potential inhibitors of COVID-19 Mpro. In a similar molecular docking study using sixty-seven molecules of natural origin, crocin, digitoxigenin and b-eudesmol were proposed as inhibitors against coronavirus[31]. Another study was carried out using one hundred seventy-one essential oil components. The study determined the best docking ligands for the SARS-CoV target proteins were (E)--farnesene, (E,E)--farnesene and (E,E)-farnesol, thereby suggesting essential oil components may act synergistically with other antiviral agents, or they may provide some relief of COVID-19 symptoms[32]. Computer modeling studies and clinical studies against SARS-CoV-2 in some prominent plants/products and their

Table 1 Antiviral effects of plants on respiratory viruses

Plant name	Preparation	Susceptible viruses	Ref.
Allium sativum (Garlic)	Aqueous extracts	Influenza A (H9N2)	Rasool *et al*[53], 2017
	Extract	Infectious bronchitis virus	Mohajer Shojai *et al*[54], 2016
	Ethanolic extract	Influenza A (H1N1)	Chavan *et al*[55], 2016
	Garlic oil	Influenza A (H1N1)	Choi[56], 2018
	Fresh extract	Influenza A (H1N1)	Mehrbod *et al*[57], 2013
	Aqueous extract	Adenovirus (ADV3 and ADV41)	Chen *et al*[58], 2011
Aloe vera (Aloe)	Aloe anthraquinones and several derivatives (3-O-tetraacetoglupiranosil)	Influenza A	Borges-Argáez *et al*[59], 2019
	Aloe-emodin	Influenza A	Li *et al*[60], 2014
Astragalus mongholicus (Astragalus)	*Astragalus* polysaccharides	Avian infectious bronchitis virus	Zhang *et al*[61], 2018
	Astragalus polysaccharide	Influenza A (H9N2)	Kallon *et al*[62], 2013
Camellia sinensis (Green tea)	Catechins -EGCG	Adenovirus	Weber *et al*[63], 2003
	Catechin	Influenza A	Kuzuhara *et al*[64], 2009
	Catechins	Influenza A (H5N1)	Liu *et al*[65], 2012
	Polyphenols	Influenza A; Influenza B	Yang *et al*[66], 2014
Curcuma longa (Turmeric)	Curcumin	Influenza A virus	Chen *et al*[67], 2013
			Dai *et al*[68], 2018
	Curcumin	Influenza A (H1N1, H6N1)	Chen *et al*[69], 2010
	Curcumin	RSV	Obata *et al*[70], 2013
Echinacea purpurea (Purple coneflower)	*E. purpurea* fresh herb and root tinctures	Influenza	Vimalanathan *et al*[71], 2013
	Standardized *E. purpurea* extract	Influenza A (H5N1, H7N7, H1N1)	Pleschka *et al*[72], 2009
	Standardized *E. purpurea* extract	Rhinoviruses, RSV	Hudson *et al*[73], 2011
Eucalyptus globulus (Eucalyptus)	Essential oil- vapor phase	Influenza	Vimalanathan *et al*[74], 2014
Ginkgo biloba (Ginkgo)	Leaf extract	Influenza A (H1N1, H3N2)	Haruyama *et al*[75], 2013
Glycyrrhiza sp. (Licorice)	Water extract of licorice (*Glycyrrhiza uralensis*)	RSV	Feng Yeh *et al*[76], 2013
	Glycyrrhizic acid derivatives	SARS-CoV	Hoever *et al*[77], 2005
	Extract of *Glycyrrhiza inflata*	Influenza A (H1N1)	Dao *et al*[78], 2011
	Glycyrrhizin	Influenza A	Wolkerstorfer *et al*[79], 2009
	Glycyrrhizin	Influenza A (H5N1)	Michaelis *et al*[80], 2010
Lepidium meyenii (Maca)	Extracted with methanol	Influenza A; Influenza B	Del Valle Mendoza *et al* [81], 2014
Melaleuca alternifolia (Tea tree)	Tea tree oil	Influenza A (H1N1)	Garozzo *et al*[82], 2011
	Aerosol and vapor of tea tree oil	Influenza A (H11N9)	Usachev *et al*[83], 2013
	Tea tree oil	Influenza A (H11N9)	Pyankov *et al*[84], 2012
Melissa officinalis (Lemon balm)	Essential oil	Influenza A (H9N2)	Pourghanbari *et al*[85], 2016
	Extract	Avian infectious bronchitis	Lelešius *et al*[86], 2019
Mentha piperita (Peppermint)	Ethanol extract	RSV	Li *et al*[87], 2017
	Extract	Avian infectious bronchitis	Lelešius *et al*[86], 2019

Nigella sativa (Black cumin)	Ethanol extracts of	Influenza A (H5N1)	Dorra *et al*[88], 2019
	Ethanol extracts of	Influenza A (H9N2)	Umar *et al*[89], 2016
	Extract	Coronavirus	Ulasli *et al*[90], 2014
Panax ginseng (Ginseng)	Root of plant *Panax ginseng*	RSV	Lee *et al*[91], 2014
	Panax Korean red ginseng extract	RSV	Lee *et al*[92], 2014
	Red ginseng extract and polysaccharide and saponin fractions	Influenza A (H1N1)	Yin *et al*[93], 2013
	Korean red ginseng extract	Influenza A (H1N1, H3N2)	Yoo *et al*[94], 2012
Pelargonium sidoides (Pelargonium)	*Pelargonium sidoides* radix extract EPs® 7630	Rhinovirus	Roth *et al*[95], 2019
	EPs® 7630	Respiratory viruses	Michaelis *et al*[96], 2011
	EPs® 7630	Influenza A (H1N1, H3N2)	Theisen *et al*[97], 2012
Sambucus nigra (Black elder)	Extract	Infectious bronchitis virus	Chen *et al*[98], 2014
	Standardized elderberry liquid extract	Influenza A; Influenza B	Krawitz *et al*[99], 2011
	Concentrated juice of elderberry	Influenza A	Kinoshita *et al*[100], 2012
	Elderberry flavonoids	Influenza A (H1N1)	Roschek *et al*[101], 2009
Scutellaria baicalensis (Chinese skullcap)	Chemical constituents	Influenza A (H1N1)	Ji *et al*[102], 2015
	Baicalin	SARS-CoV	Chen *et al*[103], 2004
Torreya nucifera (Japanese nutmeg yew)	Ethanol extract	SARS-CoV	Ryu *et al*[104], 2010
Thymus vulgaris (Thyme)	Essential oil- liquid phase	Influenza	Vimalanathan *et al*[74], 2014
	Extract	Avian infectious bronchitis	Lelešius *et al*[86], 2019
Withania somnifera (Ashwagandha)	Withaferin A	Influenza A (H1N1)	Cai *et al*[105], 2015
Zingiber officinalis (Ginger)	Aqueous extracts	Influenza A (H9N2)	Rasool *et al*[53], 2017
	Ethanol extracts	Influenza A- (H5N1)	Dorra *et al*[88], 2019
	Fresh ginger	RSV	Chang *et al*[106], 2013

Influenza A strains: H1N1, H3N2, H5N1, H6N1, H7N7, H9N2, H11N9; RSV: Respiratory syncytial virus; H1N1: Influenza A; SARS-CoV: Severe acute respiratory syndrome coronavirus.

metabolites are given below.

Curcuma longa

Utomo and Meiyanto[33] revealed the potential of several compounds of *Curcuma longa* against SARS-CoV-2 by binding to three protein receptors (*RBD-S*, *PD-ACE2*, *SARS-CoV-2 protease*). They showed that *Curcuma* sp. compounds can bind to target receptors, thus, have potential inhibitory effects on SARS-CoV-2 infectivity. Rajagopal *et al*[34] showed in their *in silico* docking study that *Curcuma longa* components could be effective against COVID-19 by inhibiting the SARS-CoV-2 Mpro enzyme. Morever, cyclocurcumin and curcumin possess significant binding at the active site of SARS-CoV-2 Mpro when compared to hydroxychloroquine and nelfinavir. When compared to remdesivir, cyclocurcumin is significantly more active [Glide score: Cyclocurcumin (−6.77); remdesivir (−6.38); curcumin (−6.13); nelfinavir (−5.93); hydroxychloroquine (− 5.47)]. In a similar study, diacetylcurcuminin was more effective on COVID-19 (Mpro) than nelfinavir[35]. Another study suggested the use of curcumin with hydroxychloroquine to destabilize the SARS-CoV2 receptor proteins[36]. Gonzalez-Paz *et al*[37] showed that curcumin strongly binds to 3CL-protease of COVID-19 Curcumin caused enzyme folding and structural changes in viral protease. Moreover, curcumin bound more strongly to the enzyme than chloroquine.

Eucalyptus globulus

Sharma[38] suggested that eucalyptus essential oil active compounds are potential inhibitors of COVID-19 Mpro. They conducted a molecular docking study to evaluate

Table 2 Human clinical studies showing the effect of plants on respiratory infections

Plant	Disease state	Participant	Dosage	Study design	Results
Aged garlic extract[107]	Cold andflu illness	120 healthy subjects, 2 groups (21-50 yr)	4 capsules/d (2.56 g); 90 d	Double-blind, randomized, placebo-controlled parallel intervention	Increase in γδ-T cell and NK cell. Reduction in cold and flu severity; decrease in symptom days
E. purpurea and *E. angustifolia* root[108]	New-onset common cold	719 patients, 4 parallel groups (12-80 yr)	First 24 h: Equivalent of 10.2 g of root. Next 4 d: 5.1 g	Randomized, controlled trial	Disease duration and severity are not statistically significantly changed
Echinacea purpurea alcohol extract (Echinaforce®) [109]	Common cold	755 healthy subjects, 2 groups (≥ 18 yr)	Illness prevention: 3 × 0.9 mL. Acute stages of colds: 5 × 0.9 mL	Randomized, double-blind, placebo-controlled trial	Reduction of the total number of cold episodes, cumulated episode days, and pain-killer medicated episodes. Inhibited virally confirmed colds and especially prevented enveloped virus infections. Maximal effects on recurrent infections. Prophylactic intake of *E. purpurea* over a period of 4 mo to provide a positive risk/benefit ratio
Echinacea root extract[110]	Respiratory symptoms	175 adults, 2 groups (18–65 yr)	Tablets: 112.5 mg *E. purpurea* 6:1 extract (equivalent to 675 mg dry root) and 150 mg *E. angustifolia* 4:1 extract (equivalent to 600 mg dry root) 3 × 1 tablet, if required: 3 × 2 tablets	Randomized, double blind, placebo-controlled trial	Lower respiratory symptom scores. Preventive effect against the development of respiratory symptoms during travel, including long-haul flights
Green tea catechins and theanine[111]	Influenza	200 healthcare workers, 2 groups	Capsules: Green tea catechins (378 mg/d) and theanine (210 mg/d). 5 m	Randomized, double-blind, placebo-controlled trial	Lower incidence of influenza infection in the catechin/theanine group
Ivy leaf extract[112]	Acute or chronic bronchial inflammatory disease	9657 patients (5181 children)	Ivy leaves extract [drug-to-extract ratio: 5-7.5:1; extraction solvent: ethanol 30% (w/w)]. 0–5 yr: 3 × 2.5 mL; 6–12 yr: 3 × 5 mL; 12 yr and adults: 3 × 5–7.5 mL. 7 d	Prospective, open, multicenter post marketing study	Healing or improvement in 95% of symptoms. Effective and well tolerated
Ivy extract (Hedelix®) [113]	Acute respiratory catarrh and/or chronic recidivating inflammatory bronchial disease	268 children, 2 groups (syrup and drops groups) (0-12 yr)	0-1 yr: 1 × 2.5 mL syrup or 3 × 5 drops, 1-4 yr: 3 × 2.5 mL syrup or 3 × 16 drops, 4-10 yr: 4 × 2.5 mL syrup or 3 × 21 drops, 10-12 yr: 3 × 5 mL syrup or 3 × 31 drops. 14 d	Independent open, non-interventional studies	Effective and safe treatment of cough. Reduction in symptoms (especially rhinitis, cough and viscous mucus)
Ivy leaves dry extract (Prospan ®) [114]	Bronchial asthma	30 children (suffering from partial or uncontrolled mild persistent allergic asthma despite long-term treatment with 400 μg budesonide equivalent), 2 groups (6–11 yr)	2 × 5 mL (corresponding to 70 mg extract) 28–30 d	Randomized, double blind, placebo-controlled, cross-over study	Improvement of MEF75-25, MEF25 and VC
Korean red ginseng extract[115]	Influenza-like illness	100 healthy adults, 2 groups (30-70 yr)	9 capsules/d. 3 m	Placebo-controlled trial	Reduced the incidence of influenza-like illness
Modified ginseng extracts (GS-3K8 and GINST)[116]	Acute respiratory illness	45 healthy applicants, 3 groups (39-65 yr)	Capsules: 500 mg; 6 capsules/d; 8 wk	Randomized, double-blind, placebo-controlled pilot study	Reduction in acute respiratory illness development and symptom duration
Panax quinquefolius extract CVT-E002[117]	Acute respiratory illness and Chronic Lymphocytic Leukemia	293 patients, 2 groups (≥ 18 yr)	2 × 200 mg extract. 3 m	Randomized, double-blind, placebo-controlled study	Reduction intense acute respiratory illness and moderately-severe sore throat. Increased antibody responses.

Panax ginseng [118]	Chronic obstructive pulmonary disease	14 participants, 2 groups (57–73 yr)	2 × 200 mg 4 wk	Clinical trial protocol and pilot study	One participant in *P. ginseng* group reported events of sore throat, cough and fever
Panax ginseng root extract [119]	Chronic obstructive pulmonary disease	168 participants, 2 groups	2 × 100 mg capsules. 24 wk	Randomized, multi-center, double-blind, placebo controlled	Reduction in symptoms
Pelargonium sidoides extract EPs® 7630 [120]	Chronic obstructive pulmonary disease	199 adults, 2 groups (18 yr and older)	30 drops. 24 wk	Randomized, double-blind, placebo-controlled, parallel group trial	Improvement in HRQoL (health-related quality-of-life) and PRO (Patient-reported outcomes)
Pelargonium sidoides extract EPs® 7630 [121]	Acute bronchitis	220 patients (1-18 yr)	1-6 yr: 3 × 10 drops; 6–12 yr: 3 × 20 drops; 12-18 yr: 3 × 30 drops; 7 d	Randomized, double-blind, placebo-controlled clinical trial	Reduction in the total score of bronchitis-specific symptoms (especially cough and rales at auscultation)
Pelargonium sidoides extract EPs® 7630 [122]	Upper respiratory tract infections	28 children with a diagnosed transient hypogammaglobulinemia of infancy (1-5 yr)	3 × 10 drops; 7 d	Randomized, placebo controlled, prospective, monocentric pilot study	Increased appetite. Reduction of nasal congestion
Pelargonium sidoides root extract EPs® 7630[123]	Upper respiratory tract- asthma attacks	61 children (1–14 yr)	1–5 yr: 3 × 10 drops; 6–12 yr: 3 × 20 drops; 12 yr and above: 3 × 30 drops; 5 d	Randomized, placebo controlled	Reduction the severity of symptoms (especially cough and nasal congestion). Shortening of the duration of upper respiratory viral infections. Reduction asthma attack frequency
Pelargonium sidoides preparation EPs® 7630 [124]	Acute non-streptococcal tonsillopharyngitis	126 children, 2 groups (6–10 yr)	3 × 20 drops. 6 d	Double-blind, placebo-controlled clinical trial	Decrease in tonsillitis severity score compared to placebo in the EPs® 7630 group after 4 d of treatment
Pelargonium sidoides extract EPs® 7630 [125]	Common cold	207 adults (18-55 yr)	SD: 3 × 30 drops; HD: 3 × 60 drops; 10 d	Prospective, double-blind, parallel-group, placebo-controlled, phase 3, 2 parts, 2-arm, clinical trial	After 10 d, clinical treatment in 90.4% of the active drug group. Reduction the severity of symptoms and short the duration of the disease. Higher full recovery rates or greater recovery for HD treatment on day 5
Sambucus nigra extract [126]	Influenza	64 patients (16-60 yr)	Lozenge: 175 mg extract; 4 lozenges/d; 2 d	Randomized, double-blind, placebo-controlled, pilot clinical trials	Significant improvement in most symptoms within 24 h (fever, headache, muscle aches and nasal congestion). Significant improvement in all investigated symptoms within 48 h (cough and mucus discharge)
Sambucus nigra extract [127]	Respiratory health	312 adults, 2 groups	Capsules: 300 mg. Before travel: 2 capsules/d. During travel and after arrival: 3 capsules/d. 14 d	Randomized, double-blind placebo-controlled clinical trial	Reduction of cold duration and severity in air travelers. Low symptom score

SD: Standard dose; HD: High dose.

the effect of eucalyptol (1.8 cineol), which is a component of eucalyptus essential oil, on Mpro. They showed that eucalyptol/Mpro complexes produce hydrophobic interactions, strong ionic interactions, hydrogen bond interactions, and eucalyptol may be a potential inhibitor of COVID-19 Mpro. Similarly, M pro/3CL pro/eucalyptol complexes have been shown to form hydrophobic interactions[39]. In another study, Sharma and Kaur[40] suggested jensenone, the component of eucalyptus essential oil, as a potential COVID-19 Mpro inhibitor. In a molecular docking study of 12 active ingredients of eucalyptus essential oil, all of these ingredients were found to bind effectively to the COVID-19 S-protein. Especially the toruatone component was effectively bound and the Spike (S) protein/Toruatone complexes formed hydrogen and hydrophobic interactions[41]. Muhammad *et al*[42], in a study of the molecular

Table 3 Plants that can have an impact on coronavirus disease 2019 symptoms

Plant name	Effects	Ref.
Allium sativum (Garlic)	Analgesic	Dehghani *et al*[128], 2018
	Anti-inflammatory	Arreola *et al*[129], 2015
	Anti-platelet	Hiyasat *et al*[130], 2009
	Heart protection	Sultana *et al*[131], 2016
	Hepatic protection	Aprioku *et al*[132], 2017
	Improving GI function	Chen *et al*[133], 2018
	Renal protection	Seckiner *et al*[134], 2014
Curcuma longa (Turmeric)	Analgesic	Henrotin *et al*[135], 2020
		Eke-Okoro *et al*[136], 2018
	Antiemetic	Liu *et al*[137], 2018
	Antifatigue	Huang *et al*[138], 2015
	Anti-inflammatory	Shimizu *et al*[139], 2019
	Antifibrotic	Gouda *et al*[140], 2019
	Antipyretic	Haider *et al*[141], 2013
	Bronchodilator	Ram *et al*[142], 2003
	GI protection	Haider *et al*[141], 2013
		Dulbecco and Savarino[143], 2013
	Hepatic protection	Dulbecco and Savarino[143], 2013
Glycyrrhiza glabra (Licorice)	Antitussives	Nosalova *et al*[144], 2013
		Kuang *et al*[145], 2018
	Anti-inflammatory	Kao *et al*[146], 2010
	Respiratory system protection	Shi *et al*[147], 2011
Nigella sativa (Black cumin)	Analgesic	Rushmi *et al*[148], 2017
	Anticoagulant	Muralidharan-Chari *et al*[149], 2016
	Antihistaminic	Ansari *et al*[150], 2010
		Alsamarai *et al*[151], 2014
	Anti-inflammatory	Majdalawieh and Fayyad[152], 2015
		Mahdavi *et al*[153], 2016
	Bronchodilation	Boskabady *et al*[154], 2010
		Salem *et al*[155], 2017
Panax ginseng (Ginseng)	Adaptogenic	Ratan *et al*[156], 2021
Pelargonium sidoides (Pelargonium)	Antitussives	Bao *et al*[157], 2015
	Secretolytic activity	Bao *et al*[157], 2015
Scutellaria baicalensis (Chinese skullcap)	Antiemetic	Aung *et al*[158], 2005
	Anti-inflammatory	Hong *et al*[159], 2013
	GI protection	Mehendale *et al*[160], 2007
		Cui *et al*[161], 2021
	Hepatic protection	Thanh *et al*[162], 2015
	Neuroprotective	Dai *et al*[163], 2013
	Regulation of histamine release-Anti allergic	Bui *et al*[164], 2017
Thymus vulgaris (Thyme)	Analgesic	Laub[165], 2018

		Salmalian *et al*[166], 2014
	Anticoagulant	Okazaki *et al*[167], 2002
	Anti-inflammatory	Habashy *et al*[168], 2018
Withania somnifera (Ashwagandha)	Adaptogenic	Salve *et al*[169], 2019
	Analgesic	Murthy *et al*[170], 2019
	Anticoagulant, antithrombotic	Ku *et al*[171], 2014
	Anti-inflammatory	Gupta and Singh[172], 2014
	Antitussives	Nosalova *et al*[144], 2013
	Stress-relieving	Lopresti *et al*[173], 2019
Zingiber officinale (Ginger)	Analgesic	Maghbooli *et al*[174], 2014
		Bartels *et al*[175], 2015
	Antiemetic	Tóth *et al*[176], 2018
	Anti-inflammatory	Khan *et al*[177], 2015
	Antiplatelet, antithrombotic	Lee *et al*[178], 2017
	Antitussives	Bera *et al*[179], 2016
	GI protection	Nanjundaiah *et al*[180], 2011
	Hepatic protection	Ajith *et al*[181], 2007
	Nephroprotective	Ajith *et al*[182], 2007

insertion of eucalyptus active ingredients into Mpro, showed that the α-gurjune of eucalyptus, aromadene and allo-aromadene components have strong binding energy.

Glycyrrhiza glabra

Sinha *et al*[43] conducted molecular docking simulation studies of two antiviral drugs (lopinavir and ribavirin) and 20 compounds of *Glycyrrhiza glabra*. Two protein targets from COVID-19 have been identified: Non-structural protein-15 endoribonuclease and spike glycoprotein. Glycyrrhizic acid prevented the virus from entering the host cell, due to its bulky structure. Gliasperin A showed high affinity to Nsp15 endoribonuclease and inhibited its activity. The authors suggested that glycyrrhizic acid disrupts the connection of the virus with the ACE2 receptor at the input level, and Gliasperin A inhibits the replication process of the virus after it enters the host cell. Another study showed that glycyrrhizin can be highly bound to Mpro[44].

Scutellaria baicalensis

Liu *et al*[45] investigated the *in vitro* effect of *Scutellaria baicalensis* and its components on COVID-19. Baicalein (its main ingredient) and the ethanol extract of the plant inhibited the 3CLpro activity and replication of COVID-19. The ethanol extract also inhibited viral entry. Udrea *et al*[46] suggested the benefit *Scutellaria baicalensis* flavones (especially baicalein) against respiratory damage caused by COVID-19. Flavones bound to 3CLpro. strongly bound to wogonin flavone, nitric oxide synthase and cyclooxygenase 2. In addition, norwogonin and baicalein arachidonate modulated 15-lipoxygenase and lysine-specific demethylase 4D analogue.

Thymus vulgaris

In a randomized clinical study conducted on patients suffering from COVID-19, it was found that *Thymus vulgaris* strengthens the immune system and can be used to reduce COVID-19 symptoms. In the study, 83 COVID-19 patients were randomly divided into the control group and the group receiving thyme (TRG). TRG was given as thyme essential oil three times a day for seven days. A questionnaire asking about symptoms such as fever, cough, fatigue, and loss of appetite was completed before and at the end of treatment to determine the effect of thyme on symptoms. Thyme essential oil significantly reduced the severity of symptoms such as fever, cough, shortness of breath, dizziness, muscle pain, anorexia, weakness and lethargy and fatigue. Additionally, thyme increased lymphocyte count and calcium while decreasing blood urea nitrogen and neutrophil count[47]. Carvacrol, a component of thyme, has been

Table 4 Angiotensin-converting enzyme inhibitor plant

Plants	The compound under study	Results	Ref.
Ammoides verticillata essential oil	Isothymol	SARS-CoV-2/ACE2 inhibition	Abdelli *et al*[183], 2021
Allium sativum essential oil	Organosulfur compounds (99.4% of its essential oil)	SARS-CoV-2/ACE2 inhibition. Garlic essential oil can prevent protein maturation of the virus and the spread of infection	Thuy *et al*[184], 2020
Apium graveolens	Apigenin	Kidneys of spontaneous hypertensive rats/Regulation in ACE2 expression	Sui *et al*[185], 2010
Camellia sinensis	Black tea; Dark tea; Green tea; Oolong tea; White tea	ACE inhibition: Green < oolong < white < black < dark teas	Dong *et al*[186], 2011
Citrus aurantium *Erigeron breviscapus* *Glycine max* *Glycyrrhiza radix* *Scutellaria baicalensis*	Hesperetin. Scutellarin. Nicotianamine. Glycyrrhizin. Baicalin	SARS-CoV-2/Connecting to ACE2 and blocking the SARS-CoV-2 input	Chen and Du[187], 2020
Geranium and lemon essential oils	Citronellol and limonene	SARS-CoV-2/ACE2 inhibition	Senthil Kumar *et al*[188], 2020
Ginseng *Glycyrrhiza uralensis*	Ginsenoside Rg6; Ginsenoside F1; Monoammonium glycyrrhizinate; Glycyrrhizic acid methyl ester	SARS-CoV-2/ACE2 kinase inhibition	Zi *et al*[189], 2020
Glycine max (soybean)	Nicotianamine	ACE2 inhibition	Takahashi *et al*[190], 2015
Glycyrrhiza glabra	Glycyrrhizic acid	SARS-CoV-2/Glycyrrhizic acid disrupts the connection of the virus with the ACE2 receptor at the entry level	Sinha *et al*[43], 2021
Hibiscus sabdariffa anthocyanins	Delphinidin- and cyanidin-3-O-sambubiosides	ACE inhibition	Ojeda *et al*[191], 2010
Linum usitatissimum (Flaxseed)	Secoisolariciresinol diglucoside	ACE inhibition	Prasad *et al*[192], 2013
Melaleuca cajuputi essential oil	Components (70.9% of the oil)	SARS-CoV-2/ACE2 and PDB6LU7 proteins inhibition	My *et al*[193], 2020
Nicotiana benthamiana	Recombinant ACE2-Fc fusion protein produced from *N. benthamiana*	SARS-CoV-2/Strong binding to the RBD of SARS-CoV-2 and inhibition	Siriwattananon *et al*[194], 2020
Withania somnifera	Withanone	SARS-CoV-2/Docking to the connector interface of the AEC2-RBD complex	Balkrishna *et al*[51], 2020

ACE: Angiotensin-converting enzyme; RBD: Receptor binding domain; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme-2.

shown to inhibit Mpro by *in silico* study. It can be a potential inhibitor of controlling viral replication[48].

Withania somnifera

W. somnifera components withanolides have potential antiviral properties on COVID-19[49]. Patel *et al*[50] demonstrated that *W. somnifera*'s Withanoside VI components have positive interactions at the binding site of protein targets of SARS-CoV-2. Withanone reduced the electrostatic interaction between ACE2 and receptor binding domain[51]. Withaferin A, which is found in the *W. somnifera* plant, has been shown to interact with Mpro and Glucose regulated protein 78 (GRP78) receptor[52].

CONCLUSION

In this study, the concept of "being effective against COVID-19" for herbal treatments was discussed from the angles of antiviral effect and control of symptoms, specifically related to GI system.

Antiviral effects on COVID-19

Since COVID-19 is a virus that mainly affects the respiratory tract, the antiviral effects of medicinal plants against respiratory viruses have been examined firstly. The structure similarities of SARS-CoV-2 have been found with SARS-CoV and Middle East respiratory syndrome coronavirus. Therefore, it can be suggested that plants and their compounds affecting these viruses may also be potential treatment options for COVID-19. Here firstly, clinical studies supporting antiviral effects of 22 plant on respiratory viruses has been reviewed which determined that glycyrrhizic acid derivatives obtained from *Glycyrrhiza* sp, *Nigella sativa*, *Scutellaria baicalensis* and *Torreya nucifera* have anti-COVID-19 effects. Plants such as *Allium sativum*, *Glycyrrhiza glabra*, *Melaleuca* sp, *Withania somnifera* have been shown to bind to ACE2 receptors that are imperative for COVID-19 replication. Focusing on these plants might be a logical way to go for herbal treatment against COVID-19.

This review also showed the antiviral effects of essential oils obtained from plants have the potential to affect COVID-19. The treatment involves using inhaled steam supplemented by essential oils possessing natural antimicrobial properties, oropharyngeal sanitization, as well as they are remedies for symptomatic relief. Inhalation of antimicrobial essential oils may help attenuate the virus in the nasal cavity, nasopharynx, oropharynx, and laryngopharynx. Antiseptic mouthwashes and gargles can also help to sanitize the oral cavity and oropharynx, whereas antiseptic lozenges can help to sanitize the oro- and laryngopharynx as well. The steam will carry the tiny particles of the antimicrobial constituents from these essential oils into the respiratory tract and is likely to improve the efficacy of the steam treatment. The steam supplemented by antimicrobial volatile oils may help to provide a local antimicrobial effect within the airways.

There are computer model studies showing that some botanicals and active ingredients are effective in COVID-19. *Allium sativum*, *Curcuma longa*, *Eucalyptus globulus*, *Glycyrrhiza glabra*, *Melaleuca* sp, *Thymus vulgaris*, *Withania somnifera* is among these plants. These studies with commonly found plants will guide future studies to develop effective supplements or drugs for COVID-19.

Symptomatic treatment of COVID-19

Since the symptoms of COVID-19 seriously affect the quality of life, herbal options to eliminate them were also evaluated in this review. Previously, herbs such as garlic, echinacea and ginseng were found to reduce the symptoms of cold in healthy individuals. Plants with their pharmacological effects are natural options for eliminating the symptoms of COVID-19. Based on the effects described in Table 3, *Allium sativum*, *Curcuma longa*, *Scutellaria baicalensis* and *Zingiber officinale* are easily found as prominent plants to eliminate the GI symptoms of COVID-19. For example, ginger can eliminate the negative effects of COVID-19 on the GI system with its antiemetic and hepatic protective properties. A clinical study was conducted with thyme essential oil on COVID-19. Thyme essential oil was found to significantly reduce COVID-19 symptoms. This revealed an option that thyme and essential oil have potential effects for consideration in treatment of COVID-19. Studies on more essential oils of eucalyptus reveal more effects of eucalyptus on respiratory system symptoms. *Eucalyptus globulus*, *Hedera helix*, *Pelargonium sidoides*, *Sambucus nigra*, *Thymus vulgaris* can be recommended for relief of respiratory symptoms. ACE2 receptors are found in tissues other than the lung, such as the intestine. Based on this fact, we concluded that the use of herbs binding to ACE2 receptors can eliminate the side effects that may occur in variety of organs including GI tract. As shown in Table 4 these plants are *Ammoides verticillate*, *Allium sativum*, *Apium graveolens*, *Camellia sinensis*, *Citrus aurantium*, *Erigeron breviscapus*, *Glycine max*, *Glycyrrhiza glabra*, *Hibiscus sabdariffa*, *Linum usitatissimum*, *Melaleuca* sp., *Nicotiana benthamiana*, *Withania somnifera*.

Based on these studies, herbal treatments offer several potential treatments of COVID-19. Plants may be an option for the treatment of COVID-19 and its symptoms, as well as protection from COVID-19. Even though these data point to good outcomes there is always the possibility of interaction between drugs used and these herbs. For instance, herbs such as ginger with antithrombotic effects can be beneficial on COVID-19 symptoms, but one might be cautious about escalated risk of bleeding when it is used together with antithrombotic or anticoagulant drugs. Therefore, it is extremely important to avoid the indiscriminate use of plants.

For a plant to be used as a medicine, its effect must be supported by clinical studies. COVID-19 is just emerging, and more research are needed for its treatment. Yet, herbal therapies are potential options for both antiviral effects and the control of COVID-19 symptoms. Since plants with multiple pharmacological effects can affect many systems

(respiratory, GI, and nervous), herbs might be more effective against COVID-19 than synthetic drugs. But first, all aspects of SARS-CoV-2 need to be examined. Then, the effects of plants on this virus should be determined by further studies.

The strengths and weaknesses of this review

Unlike other studies, in this report, the effect of plants on COVID-19 was evaluated in several ways. Preclinical studies, clinical studies and silico studies are included in this review. Moreover, the efficacy on COVID-19 symptoms has been addressed by including different systems. On the other hand, the focus is on the respiratory and GI systems. The effects, not only of botanicals but also active metabolites of have been studied.

The biggest limitation of this study is the lack of sufficient studies on the efficacy of botanicals. Since botanical studies are generally preclinical studies, results may vary due to conducting and including clinical studies. In clinical studies showing the effects of the plants in Table 2 on respiratory tract infections, the results were generally obtained with questionnaire studies. Placebo effects and breadth of study may be effective in positive results.

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MINIREVIEWS

Current and emerging therapeutic strategies in pancreatic cancer: Challenges and opportunities

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Abstract

Pancreatic carcinoma (PC) is one of the leading causes of cancer-related deaths worldwide. Despite early detection and advances in therapeutics, the prognosis remains dismal. The outcome and therapeutic approach are dependent on the stage of PC at the time of diagnosis. The standard of care is surgery, followed by adjuvant chemotherapy. The advent of newer drugs has changed the landscape of adjuvant therapy. Moreover, recent trials have highlighted the role of neoadjuvant therapy and chemoradiotherapy for resectable and borderline resectable PC. As we progress towards a better understanding of tumor biology, genetics, and microenvironment, novel therapeutic strategies and targeted agents are now on the horizon. We have described the current and emerging therapeutic strategies in PC.

Key Words: Resectable pancreatic carcinoma; Borderline resectable pancreatic carcinoma; Locally advanced pancreatic carcinoma; Adjuvant therapy; Neoadjuvant therapy; Newer advances in pancreatic carcinoma

Core Tip: An improved understanding of the natural history of pancreatic carcinoma, genetics, and tumor biology has highlighted the role of novel therapeutic strategies. However, despite recent advances in the management of pancreatic carcinoma, the

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prognosis remains poor. We have attempted to conceptualize the current therapeutic strategies in light of recent advances.

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INTRODUCTION

World over, new cases of pancreatic carcinoma (PC) add up close to three lakh each year[1,2]. There hasn't been a significant increase in the long-term survival rates, with the 5-year survival rates increasing to 5%-6% over the last 30 years, despite early detection and advances in therapeutics for pancreatic cancer[3,4]. The estimates of leading causes of cancer deaths suggest that PC may become the second, next only to lung cancer in the United States over the next decade[1].

Pancreatic ductal adenocarcinoma (PDAC) is exemplified by abundant genetic mutations, germline or acquired. Among the common ones are *CDK2NA* and *KRAS* seen in nearly 90%, *TP53* in 75%-90%, and *SMAD4/DPC4* in about 50%[5,6]. Additionally, genomic and epigenetic alterations are present, which have ignited research for targeted therapy. The desmoplastic stroma and the tumor microenvironment have been the focus of clinical explorations.

The outcomes of PC depend on the stage at diagnosis. Nearly half the cases are diagnosed as metastatic, wherein the survival ranges from 7-11 mo, at best[7,8]. In cases where the disease is non-metastatic but unresectable, there is a modest increase of survival, of nearly 6 months over the metastatic disease. The peculiarity of resectable PC lies in the poor overall survival, of approximately 2 years with adjuvant therapy. This is in stark contrast to most of the other resectable cancers.

The standard of care of resectable PC is surgery followed by adjuvant chemotherapy (CT). The benefit for this approach was established by the European Study Group for Pancreatic Cancer 1 (ESPAC-1) and the CONKO-001 trials, using 5-fluorouracil (5-FU)/leucovorin and gemcitabine respectively[7-9]. The phase III randomized PRODIGE 24 trial using 5-FU/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX) and APACT trial with nab-paclitaxel with gemcitabine changed the landscape of adjuvant therapy following their success noticed in the metastatic setting [10,11]. The role of chemoradiotherapy (CRT) in the adjuvant setting is yet to see the final statement based on the existing literature. Following the lack of survival benefit with CRT in the ESPAC-1 and European Organization for Research and Treatment of Cancer (EORTC) trials and contrasting results with two registry data showing survival benefit of CRT compared to CT, one large series compared the three modalities of systemic CT, CRT or CRT followed by CT. There was a significant survival benefit with CT and CRT followed by CT than in the CRT in patients with stage III disease. This benefit was however not seen in patients with stage I/II disease[12-16].

The management of borderline resectable pancreatic adenocarcinomas (BRPCs) has seen the emergence of adjuvant regimes in the 'neoadjuvant' or 'induction therapy' role with FOLFIRINOX and nab-paclitaxel with gemcitabine[17,18]. There is no robust data to suggest the survival benefit of these protocols so far; however, there has been demonstrable tolerability and increased resection rates. A clinical challenge has been to offer adjuvant therapy to patients receiving induction therapy. The results of phase II ESPAC-5F trial, presented at the 2020 virtual ASCO meeting, comparing four arms-frontline surgery, induction therapy with gemcitabine and capecitabine, or modified FOLFIRINOX and CRT. The study revealed similar outcomes between the frontline and induction treatment[19].

The metastatic setting is seeing numerous trials with conventional CT as well as targeted agents as the biology and tumor microenvironment, genetics, and molecular concepts are being better understood. This has led to a search for novel therapeutic strategies for managing PC. This review attempts to address the challenge faced by the practicing clinician in optimal sequencing of the available modalities in the various stages of the illness.

MANAGEMENT OF RESECTABLE PANCREATIC CANCER

A resectable adenocarcinoma does not have metastases to a distant organ or distant lymph nodes; there is no vascular involvement [characterized by absence of superior mesenteric vein (SMV) or portal vein (PV) involvement], tumor thrombosis, or venous encasement > 180°. Also, the fat planes around the celiac axis (CA), hepatic artery (HA), and superior mesenteric artery (SMA) ought to be clear[20].

Surgery

Surgery is the only treatment option that offers a cure for PDAC. Surgery aims to completely resect the tumor and achieve a microscopically negative tumor margin (R0). R0 dissection is defined as clearance of > 1 mm *i.e.*, the margin of healthy tissue around the removed tumor should be > 1 mm. The various surgical options include pancreaticoduodenectomy (Whipple's procedure) and distal pancreatectomy. Pancreaticoduodenectomy with SMA first approach is the standard of care for adenocarcinoma localized to the head of the pancreas (HOP). The surgery should involve dissection of greater than 15 lymph nodes and skeletonization of SMA down to adventitia of anterior, left lateral and posterior borders[21,22]. A sampling of paraaortic lymph nodes with an examination of the frozen section is an additional option. For PDAC involving the body and tail of the pancreas, distal pancreatectomy along with splenectomy is the treatment option. This involves dissection of greater than 15 lymph nodes[23,24].

Minimally invasive techniques for pancreatic resection beginning with laparoscopic distal pancreatectomy have been attempted. They offer advantages in the form of reduced blood loss and decreased hospital stay. However, the rate of achieving positive resection margin, morbidity, and mortality of the procedure remains the same as that of an open procedure. The use of robotic techniques in Whipple's procedure has shown reduced rates of post-procedure complications[25]. Traditionally preoperative biliary drainage has been advised for patients who present with obstructive jaundice. Recent evidence, however, points towards a higher rate of perioperative complications among those undergoing pre-operative drainage *vs* those undergoing upfront surgery[26].

The risk of developing tumor recurrence among those patients who undergo curative resection for PC varies from 69%-75% at 2 years to 80%-90% at 5 years post-surgery[27]. Tumor recurrence occurs secondary to locoregional occurrence in a majority of cases. This led to the hypothesis that the use of adjuvant therapy may reduce locoregional tumor recurrence.

Post-operative complications may reduce a patients' access to adjuvant CT and overall survival. Hence, it becomes imperative to screen patients who are at high risk of post-operative complications, like elderly patients, patients with poor performance status, or higher comorbidity profiles. Preoperative pancreatic resection score (PREPARE) and Surgical results analysis and search (SOAR) are validated and useful scoring systems for assessing the risk of developing complications post-operatively[28,29].

Adjuvant CT

The gold standard treatment for resectable PC is surgery followed by adjuvant CT. The era of adjuvant CT gained prominence when the results of the European Group for Pancreatic Cancer (ESPAC-1) trial showed significant improved median survival and 5-year survival in patients who received adjuvant CT of fluorouracil and folinic acid *vs* those who underwent surgery alone (20.1 mo *vs* 15.5 mo, respectively; $P = 0.009$). This was followed by the CONKO-001 (Charité Onkologietrial) trial, using adjuvant gemcitabine, which showed a median disease-free survival of 13.4 mo and 5-year survival of 20.7% *vs* 10.4% *vs* 6.9 mo respectively in the surgery alone group. The efficacy of these two treatment regimens was compared in the ESPAC-3 trial. Results of this study showed no survival benefit of one treatment regimen over the other, however, the treatment-related adverse effects were higher in the fluorouracil and folinic acid group.

To further improve the therapeutic outcome with adjuvant CT, a concept of combination systemic therapy has evolved. Several agents have been studied in various trials (Table 1). Among those of note are the ESPAC-4, PRODIGE and APACT studies. The ESPAC-4 trial carried out a comparison of gemcitabine *vs* a combination of gemcitabine plus capecitabine, which showed favorable overall survival benefit while using combination therapy [hazard ratio (HR): 0.82, 95% confidence interval (CI): 0.68-0.98; $P = 0.032$]. However, no significant recurrence-free survival benefit was seen in 2 years of follow-up of these patients (HR: 0.86, 95%CI: 0.73-1.02; $P = 0.082$).

Table 1 Landmark trials on adjuvant treatment in pancreatic adenocarcinoma

Study	No. of patients	Treatment arms	Median DFS in mo	Median OS in mo
GITSG[33]	43	Observation	NR	20.0
		Radiotherapy + 5-FU f/b adjuvant 5-FU	NR	10.9
ESPAC-1[34]	289	Observation	NR	15.5
		Chemoradiotherapy	NR	13.9
		5-FU/folinic acid	NR	20.1
		Chemoradiotherapy + 5-FU/folinic acid	NR	19.9
CONKO-001[9]	354	Observation	6.7	20.2
		Gemcitabine	13.4	22.8
ESPAC-3[35]	1088	5-FU/folinic acid	14.1	23.0
		Gemcitabine	14.3	23.6
ESPAC-4[36]	730	Gemcitabine	13.1	25.5
		Gemcitabine + Capecitabine	13.9	28.0
CONKO-005[30]	436	Gemcitabine	11.4	26.5
		Gemcitabine + Erlotinib	11.4	24.6
PRODIGE 24-PA6[37]	493	Gemcitabine	12.8	35.0
		FOLFIRINOX	21.6	54.4
APACT[11]	866	Gemcitabine	18.8	36.2
		Gemcitabine + nab-paclitaxel	19.4	40.5

5-FU: 5-Fluorouracil; CONKO: Charité Onkologie; DFS: Disease-free survival; ESPAC: European Group for Pancreatic Cancer; GITSG: Gastrointestinal Tumor Study Group; NR: Not reported; OS: Overall survival; PRODIGE: Partenariat de Recherche en Oncologie DIGEstive.

The Partenariat de Recherche en Oncologie Digestive (PRODIGE 24-PA6) trial highlighted the successful use of FOLFIRINOX (oxaliplatin + irinotecan + leucovorin) *vs* gemcitabine in patients with good performance status (Eastern Cooperative Oncology Group, ECOG: 0-1). The median disease-free survival in patients with combination therapy was 21.6 mo *vs* 12.8 mo in the gemcitabine group. The latest in series is the Nab-paclitaxel and Gemcitabine *vs* Gemcitabine Alone as Adjuvant Therapy for Patients with Resected Pancreatic Cancer (APACT) study, which has shown encouraging results of using combination therapy of gemcitabine plus nanoparticle albumin-bound paclitaxel (nab-paclitaxel). Median disease-free survival was not statistically significant in the two arms (19.4 mo in combination arm *vs* 18.8 mo in gemcitabine only arm). Overall survival favored the combination CT group (HR: 0.82, 95%CI: 0.68-0.996; $P = 0.045$)[27].

Another concept is the use of targeted agents (erlotinib or sorafenib) or immunotherapy (algenpantucel-L) in combination with gemcitabine. However, to date, none of these agents have shown any favorable survival outcome *vs* the use of gemcitabine alone[30-32].

Adjuvant CRT

Adjuvant CRT aims to prevent locoregional tumor recurrence. Amongst the first trials to assess the efficacy of CRT in PC was the EORTC trial, which showed no significant survival benefit amongst patients of PDAC who received CRT (40 Gy + continuous infusion of fluorouracil)[13]. This was followed by the ESPAC-1 trial, which employed the following three different adjuvant therapy designs: CRT, CT alone, and CRT followed by adjuvant CT. Patients undergoing adjuvant CT were found to have poor survival benefits after a median follow-up of 47 mo (HR: 1.28, 95%CI: 0.99-1.66; $P = 0.05$)[34]. The RTOG 9704 trial was carried out to assess the benefit of adding gemcitabine to postoperative radiation + fluorouracil *vs* adjuvant therapy with fluorouracil. The trial showed no survival benefit in either of the two treatment groups [38].

Although these trials failed to prove any survival benefit, they nevertheless provided useful information on the feasibility and tolerability of these treatment options. Analysis of the US National Cancer Database which included patients of pancreatic adenocarcinoma who underwent curative resection followed by adjuvant CT showed survival benefit (median overall survival with CT + radiation: 22.3 mo and adjuvant CT alone: 20.0 mo; $P = 0.001$)[14].

Timing of adjuvant CT

There are definite gaps in our knowledge regarding the optimal timing of initiating CT following surgery and follow-up of patients undergoing adjuvant therapy for curable PC. Delay in initiating as well as non-initiation of adjuvant treatment is not uncommon. Post-operative morbidity adversely impacts the initiation of adjuvant treatment. It has been estimated that approximately 20% of patients become ineligible for adjuvant CT. The ESPAC-3 trial aimed to analyze the outcome among those who were initiated on CT within 8 wk of surgery and those who were initiated on CT after 8 wk of surgery. There was no survival benefit in either of the two arms, while successful completion of six cycles of CT was found to be an independent predictor of survival[39]. Similar results have been put forward by analyzing multi-institutional retrospective data of patients who had undergone curative resection for PDAC. Thus, the evidence so far suggests that patients who receive adjuvant therapy more than 12 wk following surgery are still good candidates for adjuvant CT[40].

Therefore, in summary, the standard of care in resectable pancreatic adenocarcinoma is surgical resection followed by adjuvant CT. Single-agent (5-FU) is preferred for periampullary tumors of pancreatic origin. Patients with adenocarcinoma in the head, body, or tail of the pancreas may be treated with FOLFIRINOX (patients with good post-operative performance status, ECOG: 0-1) and combination therapy of gemcitabine+ capecitabine in those with poor performance status postoperatively (Figure 1).

Neoadjuvant therapy

Conceptually, the use of neoadjuvant therapy in resectable PC gained prominence when this approach was shown to have improved survival benefit in other gastrointestinal malignancies[41]. It offers the following theoretical advantages: reduction of circulating tumor cells and micrometastasis before surgery, and avoiding surgery in those who experience disease progression while on neoadjuvant therapy, thus, reducing surgical mortality and morbidity. Of particular interest is the ability of neoadjuvant treatment to achieve tumor downsizing or downstaging (lower T and N stages, reducing the rates of vascular, lymphatic, and perineural invasion, and the ability to achieve better R0 resection post-surgery). Moreover, individuals who receive neoadjuvant CT are more likely to be able to access the entire therapeutic sequence[42, 43].

The preoperative or postoperative CT for resectable pancreatic adenocarcinoma (PACT-15) trial has shown the efficacy of neoadjuvant CT in patients with resectable PDAC[44]. One of the first published phase III trials assessing the role of neoadjuvant gemcitabine followed by gemcitabine and radiation before surgery is the PREOPANC trial. Although it has not shown statistically significant survival benefit in patients using neoadjuvant CT + CRT (overall survival of 16 mo for neoadjuvant therapy *vs* 14.3 mo for initial surgery), better R0 resection rates, locoregional disease-free survival, and lower rates of vascular and perineural invasion favor neoadjuvant CT[45]. Analyses of 'US National Cancer Database data have shown a favorable survival using perioperative CT in patients with early PC as compared with upfront surgery. Another similar cohort study by Mokdad and colleagues[46] demonstrated statistically significant median overall survival in the neoadjuvant group *vis a vis* upfront surgery group (26 mo *vs* 23 mo respectively; $P = 0.01$). Many other phase III trials are being conducted to have a better understanding of this therapeutic aspect[27] (Table 2). Neoadjuvant treatment is well tolerated. The risk of postoperative pancreatic fistula formation (3%-11%), risk of postoperative infections (3%-7%) and mortality (0%-4%) compared to those who have undergone surgery alone[47].

MANAGEMENT OF BRPC

The National Comprehensive Cancer Network has defined BRPC based on the following radiological criteria: Contrast-enhanced computerized tomographic (CECT) scan using the pancreatic protocol, the relationship of tumor with the surrounding

Table 2 Landmark trails on use of neoadjuvant chemotherapy

Study	No. of patients	Treatment arms	Resection rate, %	R0	Median DFS in mo	Median OS in mo
PACT-15[44]	93 Resectable	Surgery + gemcitabine	85	27	4.7	20.4
		Surgery + 6 PEXG	90	37	12.4	26.4
		3PEXG + surgery + 3 PEXG	84	63	16.9	16.9
PREOPANC-01[48]	248 Resectable + BRPC	26Gy/15fr + gemcitabine	60	63	9.9	17.1
		Surgery	72	31	7.9	13.7

BRPC: Borderline resectable pancreatic cancer; DFS: Disease-free survival; OS: Overall survival; PEXG: Cisplatin, epirubicin, gemcitabine, and capecitabine.

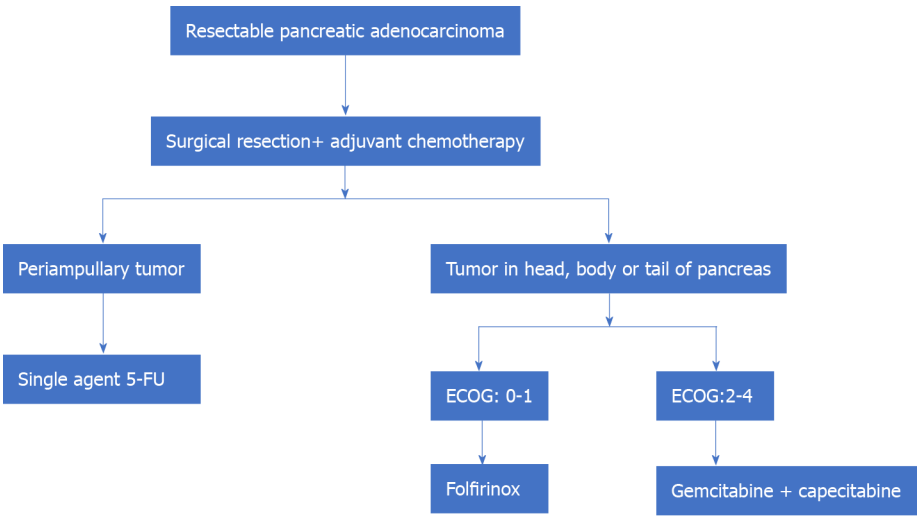

Figure 1 Management of resectable pancreatic adenocarcinoma. 5-FU: 5-Fluorouracil; ECOG: Eastern Cooperative Oncology Group.

vasculature, and the absence/presence of metastasis.

Thus, a BRPC localized to the HOP is one where the tumor is in contact with the common HA but without extension to the CA or artery bifurcation and variant arterial anatomy, contact with the SMA < 180°, contact with the SMV or PV < 180° without venous contour irregularity or thrombosis, which allows for safe and complete arterial and venous resection and reconstruction[20].

Borderline resectable PDAC in the pancreatic body or tail is 'the solid tumor in contact with the CA of < 180° or contact with the CA > 180° without the involvement of the aorta and gastroduodenal artery to allow a 'modified Appleby surgery'[20]. Another definition, the Anderson classification for BRPC, classifies it into the following three different groups: Group A includes patients with a tumor that abuts visceral arteries or causes short-segment occlusion of SMV; group B, have findings suggestive of metastasis; and group C patients are those who have marginal performance status[49].

Historically, therapeutic options for BRPC consist of upfront surgery, surgery followed by adjuvant CT/CRT, and neoadjuvant CT. The standard surgical options remain Whipple procedure, total pancreatectomy, or distal pancreatectomy, based on the tumor localization. An approach favoring upfront surgery carries with itself the risk of early failure, which has often been attributed to the poor pre-operative staging of tumor radiologically, inability to carry out a radical surgery, and the aggressive tumor behavior owing to variations in tumor biology. This has brought about an interest in considering neoadjuvant CT for patients with BRPC.

A meta-analysis carried out by the Dutch Pancreatic Cancer Group has shown a median survival of 19.2 mo in patients undergoing neoadjuvant CT *vs* 12.8 mo in those undergoing upfront surgery[50]. A peculiar problem in comparing different groups arises when different CT/CRT regimens are being compared. A recent patient-level meta-analysis has analyzed the efficacy of neoadjuvant FOLFIRINOX in BRPC, finding a favorable trend using FOLFIRINOX (median overall survival of 22 mo and median disease progression-free survival of 18.0 mo)[18].

A recent randomized control trial has assessed the use of CRT as neoadjuvant therapy for BRPC. The investigators combined 54 Gy in 30 fractions + weekly gemcitabine followed by adjuvant gemcitabine. Results have shown a better resection rate in the neoadjuvant group *vs* the surgery only group (51.8% *vs* 26.1% respectively) as well as a statistically significant 2-year survival (40.7% *vs* 26%; $P = 0.028$)[51]. The preoperative CRT *vs* immediate surgery for resectable and borderline resectable pancreatic cancer (PREOPANC-1) trial has used intention-to-treat analysis to study the efficacy of neoadjuvant CRT (gemcitabine-based) *vs* upfront surgery in BRPC. Patients in the neoadjuvant CRT group had a lower resection rate (60%) *vs* those in the upfront surgery group (72%; $P = 0.065$). R0 resection rate was statistically higher in the neoadjuvant group *vs* the upfront surgery group (61% *vs* 31%; $P < 0.001$). To add to the benefits, patients receiving neoadjuvant CRT had a longer median time till recurrence *vs* the surgery only group (9.9 mo *vs* 7.9 mo; $P = 0.023$)[48].

Results of these trials have shown the efficacy of both neoadjuvant CT as well as for CRT in BRPC. Several ongoing trials are being carried out to study the efficacy of induction therapy in improving overall survival in BRPC and which of the two therapeutic strategies (CT/CRT) is better suited for the same.

MANAGEMENT OF LOCALLY ADVANCED PC

The concept of locally advanced PC (LAPC) has evolved based on the development of radiological criteria of resectability and the availability of neoadjuvant therapy. Practically, the pancreatic tumors which are not metastatic and are unresectable due to 'irreversible' vascular invasion (encasement of aorta, invasion of PV or SMV, involvement of the SMA or celiac trunk by > 180°) are considered as LAPC[52,53]. The median survival has been variably reported from 10 mo to 30 mo.

The standard treatment for LAPC is gemcitabine-based CT. For patients with good performance status (ECOG0-1), the FOLFIRINOX-based regimen and for those with ECOG0-2, the nab-paclitaxel-gemcitabine-based regimen may be considered[53]. Some observational studies and pooled analyses of different approaches have advocated induction treatment with either FOLFIRINOX or nab-paclitaxel–gemcitabine followed by CRT. Using this approach median survival of 24.2 mo and disease progression-free survival of 15 mo has been reported[54]. However, the use of either upfront radiation therapy or following induction treatment with gemcitabine is not beneficial for patients with LAPC. Recently published meta-analysis has reported similar overall survival and drug-related side effects of CRT and CT in the setting of LAPC[55]. CRT using capecitabine as a radiosensitizer, however, has been tried for improvement of local control of disease in a subset of patients with LAPC. Since this is not a standard treatment approach, it has been proposed that this may be offered to only a select group of individuals[56].

Ongoing trials in LAPC are trying to assess the efficacy of FOLFIRINOC *vs* gemcitabine as induction therapy (NEOPAN; NCT02539537), use of nab-paclitaxel-gemcitabine for induction regimen + radiation therapy *vs* continuous CT, and use of activation of the *DPC4* gene (RTOG 1201; NCT01921751).

An interesting recent concept that has emerged in the management of LAPC is the role of surgery. This was proposed by researchers from the Medical College of Wisconsin based on the observation that those with an initially unresectable disease as per radiological criteria may convert to the resectable tumor after induction CT ± CRT. They have proposed the classification of patients with LAPC into the following two distinct categories: Type A, tumors that may be considered for resection following induction CT; and type B: Definitively unresectable tumors (> 270° encasement of the SMA, > 180° encasement of the CA, encasement of the aorta, and > 180° encasement of the HA with extension beyond the bifurcation of the proper HA into right and left HA)[52]. Thus, patients with LAPC type A, who have completed induction CT should be considered for surgical exploration. This is especially pertinent as the specificity of a CECT scan to determine tumor staging, operability, and R0 resectability for HOP carcinoma decrease following induction therapy. In such a setting, surgical exploration is the ideal modality to prove/ rule out vascular involvement[57]. Resected patients who have received CT preoperatively can also be considered for an additional course of CT following surgery[58] (Figure 2).

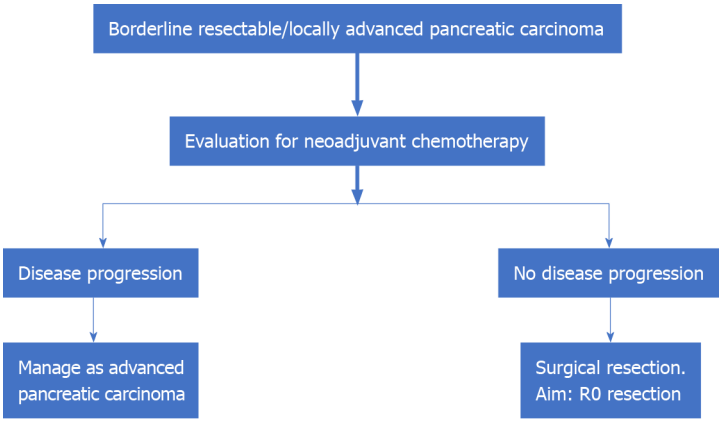

Figure 2 Management of borderline resectable/locally advance pancreatic carcinoma.

MANAGEMENT OF ADVANCED METASTATIC PC

CT forms the backbone of therapeutic regimens for the management of metastatic PC. Till the advent of gemcitabine, fluorouracil was the only approved drug in the management of APC. The use of gemcitabine has brought about benefits of disease progression-free survival, and overall survival (from 4.4 mo to 5.7 mo, $P = 0.0025$) with similar drug-related side effects as compared to fluorouracil[59]. This was followed by trials that analyzed the survival benefit of adding another cytotoxic drug or targeted therapy to gemcitabine. Use of erlotinib to gemcitabine-based regimen has shown median overall survival benefit (5.91 mo to 6.24 mo, $P = 0.038$) and 1-year survival benefit (17% to 23%, $P = 0.023$)[60]. A combination regimen consisting of cisplatin, epirubicin, fluorouracil, and gemcitabine (PEFG) *vs* gemcitabine has shown 'four-month progression-free survival' of 60% *vs* 28% in gemcitabine alone arm, $P = 0.001$ with no difference in overall survival[44]. A combination of gemcitabine + nab-paclitaxel in patients with ECOG0-2 has shown an improvement of 'median overall survival' *vs* gemcitabine alone (8.5 mo *vs* 6.7 mo, $P < 0.001$). The PRODIGE 4-ACCORD11 trial is a landmark trial that has shown the overall survival benefit of using FOLFIRINOX (median survival: 11.1 mo) in patients with APC *vs* gemcitabine (6.8 mo, $P < 0.001$)[10]. FOLFIRINOX has not been compared to nab-paclitaxel + gemcitabine in any prospective trial to date. Thus, the FOLFIRINOX regimen is now the standard of care for patients of APC with ECOG: 0-1, normal serum bilirubin, and no underlying cardiac pathology. The limiting step in the FOLFIRINOX regimen happens to be the performance status of the individual and comorbidity profile with the elderly or low-profile patients likely to have a poorer outcome. Other prognostic factors are the number of metastases, and liver metastases, presence of genetic mutations such as *DNA damage response* (*DDR*) gene mutations and *BRCA* tumor suppressor gene mutations[61-63]. Modified FOLFIRINOX has been tried with similar efficacy and better tolerance profile as compared to the standard FOLFIRINOX regimen. This regimen includes fluorouracil bolus suppression or a dose reduction of irinotecan (or both)[64].

Progress of disease in patients with first-line CT regimens presents a particular challenge with around 50% of patients being eligible for second-line CT[65]. Combination regimens like gemcitabine-platinum and fluoropyrimidine-platinum have shown disease progression-free survival of 2.5 mo *vs* 1.9 mo for single agents ($P = 0.169$) but no improvement in overall survival (5.1 mo *vs* 4.3 mo, $P = 0.169$)[66]. The various combination regimens that have been tried on the failure of first- and second-line regimens are oxaliplatin, folinic acid, and 5-FU (OFF regimen), and nanoliposomal irinotecan (MM-398), 5-FU, and folinic acid regimen. The CONKO-003 trial has shown overall survival benefit of using the OFF regimen over the 5-FU–folinic acid (FF regimen); the median survival was 5.9 mo *vs* 3.3 mo respectively ($P = 0.01$)[67]. The disease progression-free survival with use of nanoliposomal irinotecan (MM-398), 5-FU, and folinic acid regimen after failure of the FOLFIRINOX regimen has been reported to be 5.1 mo with overall survival of 8.8 mo[68]. The results of using targeted agents, namely the Jak1 and Jak2 tyrosine kinase inhibitor ruxolitinib and glufosfamide, have been rather disappointing. However, patients with metastatic solid tumors (including 8 patients with pancreatic tumors), with deficient mismatch repair and failed first-line therapy have shown response to the PD-1 immune checkpoint

inhibitor (ICI) pembrolizumab (disease control rate: 77%, objective response: 53% and complete radiological recovery: 21%)[69]. Mutation in the *BRCA* gene has been reported in around 5% of patients with APC. Targeted therapy in patients with *BRCA* gene mutation using 'poly ADP-ribose polymerase (PARP) inhibitors' is being actively investigated[70] (Figure 3).

Palliative treatment

Palliative treatment aims to allay patients' symptoms and improve their quality of life. Pain management, symptomatic relief, and psychological support are the pillars of this strategy.

Gastric outlet obstruction (GOO), extrahepatic biliary obstruction (EHBO), and abdominal pain are the three most common disabling symptoms in APC which adversely affect an individual's quality of life besides being a major source of 'caregiver fatigue'. GOO, presenting as nausea, vomiting, dehydration, and weight loss, is seen in 10%-25% of all APC cases. Palliative surgery with open gastrojejunostomy (GJ) is the traditional approach to managing a malignant GOO. Placement of endoscopic duodenal stents and laparoscopic GJ has been tried, with varying degrees of success. Surgical procedures offer good functional outcomes at the cost of increased mortality[71]. EHBO can present with obstructive jaundice. Endoscopic retrograde cholangiopancreatography (ERCP)-guided biliary stent placement is the accepted gold standard approach for the management of malignant EHBO. Both plastic and self-expanding metal stents (SEMSs) have been used, with literature favoring the use of covered SEMSs. Failure of ERCP-guided biliary drainage may warrant drainage through the percutaneous route or an endoscopic ultrasonography-guided biliary drainage (EUS BD)[72]. Hepaticojejunostomy with a Roux-en-Y reconstruction and cholecystectomy is the favored palliative surgical procedure for palliation of EHBO secondary to PC. Laparoscopic biliary bypass and robot-assisted laparoscopic hepaticojejunostomy have been technically successful with satisfactory surgical outcomes[73,74]. Thus, patients with good performance status may benefit from a palliative surgical procedure while those with poor performance status warrant endoscopic biliary drainage.

Malignant infiltration of celiac or mesenteric nerve plexus in patients of APC may cause abdominal and back pain. This may adversely affect an individual's quality of life. Multimodal drug therapy encompassing the use of non-steroidal anti-inflammatory drugs and or opioid analgesics in various combinations form the bedrock of pain management. Intraoperative celiac block using ethanol or a local anesthetic using either laparoscopic or open approach and EUS-guided neurolysis of celiac plexus, have all been tried[71] (Figure 4).

NEWER MODALITIES FOR PANCREATIC CANCER

Immunotherapy for pancreatic cancer

The tumor microenvironment of PC is responsible for aggressiveness as well as chemoresistance. This makes the case for utilizing immunotherapy in the advanced and metastatic settings. However, the approval for ICI is currently for patients with mismatch repair-deficient cases[69]. The updated results of the Keynote-158, using pembrolizumab in patients with advanced PDAC revealed an overall response rate (ORR) of 18.2%, median progression-free survival of 2.1 mo, and median overall survival of 4.0 mo[75]. The results are not very encouraging despite this, due to fewer driver mutations, variable expression of PD-1/PD-L1 and neoantigen burden in the tumor tissue, and absence of DDR, which are hallmarks of this malignancy[76].

Targeted therapy

The highly actionable mutations detected in molecular profiling of the Know Your Tumor initiative were 27%, of which the common ones involved the *KRAS, TP53, MLL3, CDKN2A, SMAD4, TGFBR2, ARID1A,* and *SF3B1* genes. These mutations, however, do not have any therapeutic modality to target[77,78]. *Neurotrophic receptor tyrosine kinase* (*NTRK*) gene fusions have been detected in about 6% of pancreatic adenocarcinomas. Larotrectinib and entrectinib are two newer agents receiving accelerated approval for use in metastatic tumors with *NTRK* gene biomarker with tissue agnostic indication[79,80]. Three single-arm trials, namely LOXO-TRK-14001, SCOUT and NAVIGATE, had a total of 55 patients with *NTRK* fusions and had an ORR of 55% with Larotrectinib[81]. The median progression-free survival was not achieved after a median duration of 9.9 mo. Similarly, in three other single-arm trials,

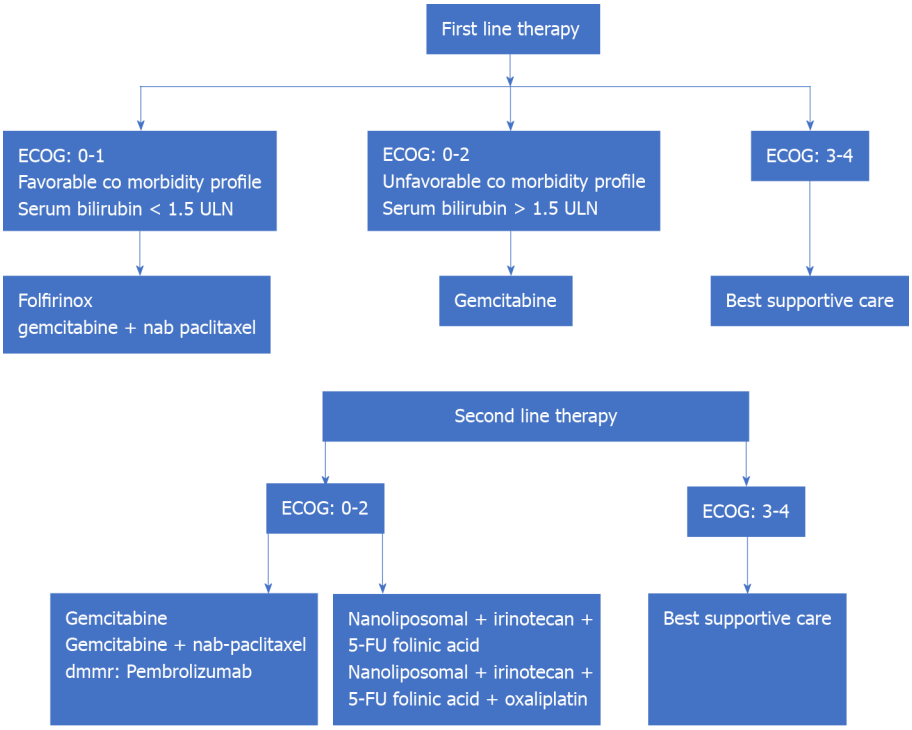

Figure 3 Management of advanced metastatic pancreatic carcinoma. ECOG: Eastern Cooperative Oncology Group; ULN: Upper limit of normal.

including ALKA-372-001, STARTRK-1 and STARTRK-2, with 60 patients in total, the patients with *NTRK* fusion had an ORR of 100%[82].

Germline *BRCA1/2* mutations and homologous recombination deficiency enable the utility of platinum agents as well as poly (adenosine diphosphate-ribose) polymerase (PARP) inhibition as a novel therapeutic modality. Olaparib, a PARP inhibitor was approved by the United States' Food and Drug Administration in December 2019 for patients with advanced metastatic malignancy having germline *BRCA1/2* mutation. The phase III POLO trial utilizing olaparib in PAC, progressing after platinum-based therapy revealed median progression-free survival of 7.4 mo *vs* 3.8 in the control arm (HR: 0.53, 95%CI: 0.35-0.82; P = 0.004). This did not translate into an overall survival benefit[83].

Macrophage-targeted therapy

Macrophages residing in the tumor environment are labeled as tumor-associated macrophages. CD 51 is a marker of macrophages that promotes the stemness of PDAC cells by regulating the TGF-β1/smad2/3 pathway. As a result, CD 51- targeted therapy is evolving as a newer therapeutic modality. Similarly, CD 40 activation, which promotes anti-tumor T-cell responses has been targeted by using anti-CD 40 antibody, CP-870893 along with gemcitabine, providing initial results of response[84].

Cancer vaccines

The ability of cancer vaccines to stimulate dendritic cell responses and activate the adaptive immune responses has been harnessed for many cancers, including PC. The expression of murine enzyme alpha-1,3-galactosyltransferase (alpha-GT) by genetic engineering on PC cell lines HAPa1 and HAPa2 lead to anti-alpha-Gal antibody responses in humans[85]. Algenpanteucel-L was used in a phase II study and phase III (IMPRESS Trial). When algenpanteucel-L was given after gemcitabine and 5-FU based CRT, 81%-86% 1-year disease-free survival and 96% 1-year overall survival were observed[86,87].

GVAX, a line of engineered pancreatic tumor cells secreting GM-CSF has been tested in phase I study along with cyclophosphamide (Cy) with early results of tolerability and survival. In a phase II study, GVAX/Cy was tested against a GVAX/Cy followed by CRS 207 (a live-attenuated *Listeria* strain that induces tumor-associated antigens) and resulted in better overall survival in the latter arm (6.1 mo *vs* 3.9 mo, P = 0.02)[88].

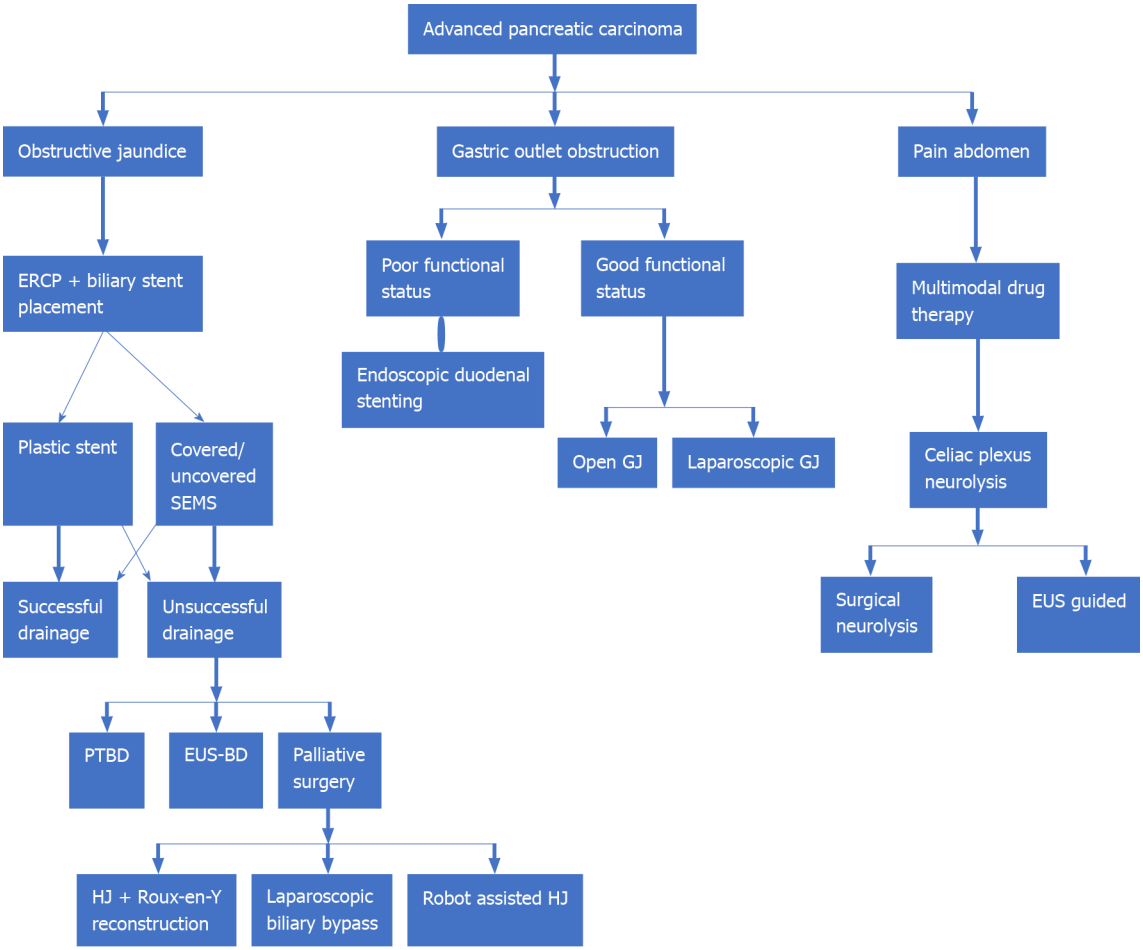

Figure 4 Palliative management in metastatic pancreatic carcinoma. ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; EUS-BD: Endoscopic ultrasound guided biliary drainage; GJ: Gastrojejunostomy; HJ: Hepaticojejunostomy; PTBD: Percutaneous transhepatic biliary drainage; SEMS: Self-expanding metal stent.

Other membranous and intracytoplasmic targets

There have been numerous trials using targets like vascular endothelial growth factor (VEGF) and VEGF-receptor, RAS-RAF-MEK-ERK pathway, *etc.* but none have shown robust survival data[89,90]. The rapamycin-insensitive companion of mTOR (RICTOR) expression was found to have a survival benefit in resected PAC patients, with those having a lower expression doubling the survival as compared to the high expressers [91].

CONCLUSION

We have attempted to provide an inclusive version of the management of PC, with special emphasis on current strategies and the road ahead with emerging modalities of therapy. Of course, there are certain gaps in the understanding of this disease and the evolution of treatment options is always challenging. For times to come, newer modalities appear promising; however, there is no substitute for early diagnosis and management for disease-free survival.

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MINIREVIEWS

Cathepsin L, transmembrane peptidase/serine subfamily member 2/4, and other host proteases in COVID-19 pathogenesis – with impact on gastrointestinal tract

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Abstract

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) seems to employ two routes of entrance to the host cell; *via* membrane fusion (with the cells expressing both angiotensin converting enzyme 2 (ACE2) and transmembrane peptidase/serine subfamily member 2/4 (TMPRSS2/4)) or *via* receptor-mediated endocytosis (to the target cells expressing only ACE2). The second mode is associated with cysteine cathepsins (probably cathepsin L) involvement in the virus spike protein (S protein) proteolytic activation. Also furin might activate the virus S protein enabling it to enter cells. Gastrointestinal tract (GIT) involvement in SARS-CoV-2 infection is evident in a subset of coronavirus disease 2019 (COVID-19) patients exhibiting GIT symptoms, such as diarrhea, and presenting viral-shedding in feces. Considering the abundance and co-localization of ACE2 and TMPRSS2 in the lower GIT (especially brush-border enterocytes), these two receptors seem to be mainly involved in SARS-CoV-2 invasion of the digestive tract. Additionally, *in vitro* studies have demonstrated the virions capability of infection and replication in the human epithelial cells lining GIT. However, also furin and cysteine cathepsins (cathepsin L) might participate in the activation of SARS-CoV-2 spike protein contributing to the virus invasiveness within GIT. Moreover, cathepsin L (due to its involvement in extracellular matrix components degradation and remodeling, the processes enhanced during SARS-CoV-2-induced inflammation) might be responsible for the dysregulation of absorption/digestion functions of GIT, thus adding to the observed in some COVID-19 patients symptoms such as diarrhea.

Key Words: COVID-19; SARS-CoV-2; Angiotensin converting enzyme 2; Transmembrane peptidase/serine subfamily member 2/4; Cathepsin L; Gastrointestinal tract

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Core Tip: Gastrointestinal tract (GIT) is believed to participate in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) dissemination. The current research shows the abundance and co-localization of angiotensin converting enzyme 2 (ACE2) and transmembrane peptidase/serine subfamily member 2 receptors in the lower GIT. Furthermore, about half of coronavirus disease 2019 patients present with GIT symptoms, such as diarrhea, and exhibit viral-shedding in feces. Additionally, *in vitro* studies have demonstrated the virions capability of infection and replication in the human epithelial cells lining GIT. This paper reviews the possible routes of the virus infection with respect to the host-enzymatic systems responsible for the proteolytic priming of SARS-CoV-2.

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INTRODUCTION

Coronaviruses may employ several host proteases for their invasion into target cells. The enzymes participating in the viruses activation include: proprotein convertases (PCs) (mainly furin), transmembrane serine proteases, especially transmembrane peptidase/serine subfamily member 2 (TMPRSS2), the lysosomal cathepsins (mainly cathepsin L), elastase, and coagulation factor Xa[1].

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus responsible for coronavirus disease 2019 (COVID-19) pandemic exhibits many similarities when compared to SARS-CoV. It employs a similar mechanism of host cells invasion; recognizes and binds the same type of angiotensin converting enzyme 2 (ACE2) receptors to enter host cells (but with higher affinity[2]), and comparable processing of spike protein (S protein) seems to be necessary for the virion fusion with the host cell membrane[3-7] (Figure 1).

Two proteolytic events need to be conducted for SARS-CoV-2 activation; initially spike protein is cut in the specific cleavage site between S1 and S2 domain, then the second cleavage within S2 domain (S2′ site) allows for the exposition of the fusion peptide, which enables membrane fusion. The first proteolytic step can happen in the producer cell, in the extracellular space, or within the host cell's endosome. This cleavage site in SARS-CoV-2 spike protein is recognized by various proteases, including furin (unlike in SARS-CoV lacking furin-cleavage site between S1 and S2)[6, 8] and TMPRSS2[3]. The second cleavage can be either performed by TMPRSS2 on the surface of the host cell, or in the endolysosomes by lysosomal proteases, most probably cathepsin L[9].

Therefore, as proposed by Pislar *et al*[10], two routes of SARS-CoV-2 entry to the host cell are likely; *via* membrane fusion with the host cells which expose both ACE2 and TMPRSS2 (and/or other transmembrane serine proteases such as TMPRSS4[11]) proteins, or *via* receptor-mediated endocytosis (RME) to the target cells expressing only ACE2 receptors. In the first case, both processing steps performed by TMPRSS2 before the virus entry enable membrane fusion, whereas in the second mechanism, the virion binding with ACE2 receptors induces endocytosis followed by spike protein activation by cathepsin L (and/or other cysteine cathepsins)[4,10]. As a result of either of these pathways, viral RNA is released in the host cell and undergoes the processes of replication (Figure 2).

TYPES OF HOST PROTEASES IN SARS-CoV-2 INVASION

The findings of several studies support the notion that, apart from ACE2 receptors, the main host peptidases involved in SARS-CoV-2 spike protein processing include: TMPRSS2 (and/or TMPRSS4), lysosomal cysteine cathepsins (mainly cathepsin L), as well as furin-like PCs. They may participate in SARS-CoV-2 activation independently,

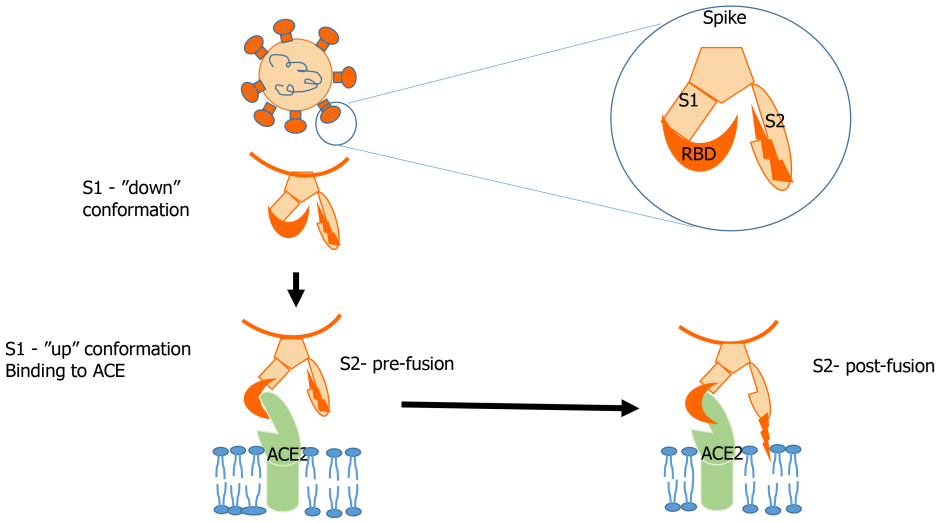

Figure 1 Alternations in spike proteins conformation upon binding to ACE 2. Spike S1 subunit contains receptor binding domain, that has to change from "down-conformation" state to "up-conformation" state to be accessible for ACE2. Changes in S1 subunit trigger conformational changes in S2 subunit, causing exposition of hydrophobic domain, changing it from "pre-fusion" to "post-fusion" state. This enables fusion of the virus with host membrane (after Zhu *et al*[7]).

or their actions may overlap or complete one another, depending on the pattern of the virion-recognized proteins exposed on the host cells (*e.g.* expressing or not TMPRSS2).

Cysteine cathepsins (CCs) in pathology

Cysteine cathepsins belonging to the papain-like family of cysteine proteases (containing cysteine in their catalytic center) comprise 11 cathepsins (B, C, F, H, K, L, O, S, V, X and W) in the human organism[12,13]. They belong to lysosomal proteases involved mainly in intracellular protein breakdown, antigen processing, MHC-II mediated immune response, and apoptosis. However, their functions go far beyond this; they participate in various physio-pathological processes not only intracellularly, but also in the extracellular matrix (ECM), because, except for their endolysosomal sequestration, they have been observed in the nucleus, cytosol, mitochondria, at the plasma membranes and in the extracellular milieu[13,14]. Their secretion is observed in physiological conditions (*e.g.* in bone remodeling – conducted by osteoclasts-secreted cathepsin K, in wound healing performed by keratinocytes-secreted cathepsin B, in prohormone processing – thyroid hormones released from thyroglobulin by cathepsins B, L and K secreted from the thyroid epithelial cells). However, an excessive secretion of cysteine cathepsins is mostly observed in pathological states associated with inflammatory processes, such as cancer diseases (cathepsins B, C, K, L, S, H, X), cardiovascular diseases (cathepsins C, K, L, S, V), joint and bone diseases (cathepsins K, B, L, S, H), inflammatory bowel disease (cathepsin L), and many other disorders (summarized in[12-15]). In these pathological states CCs typically act extracellularly, where they participate in collagen, elastin, and other ECM components degradation directly or indirectly (activating other proteases) after being secreted from recruited immune cells (mostly), as well as from inflamed tissue cells (to the lesser extent). Macrophages and other immune cells infiltrating tissues seem to be the main extracellular source of CCs whose secretion is stimulated by inflammatory factors like cytokines. However, also other cell types secrete excessive amounts of CCs, including osteoclasts or chondrocytes (oversecreting cathepsin K and/or S in arthritis and osteoporosis), or cancer cells and tumor-associated fibroblasts (oversecreting cathepsin S, L and/or B in cancer invasion)[14]. Apart from degradation of main components of ECM, also more refined processing of ECM (undergoing both intra-, and extracellularly) is ascribed to CCs. This comprises modifying and shedding cell adhesion molecules and cell membrane receptors, which affects signal transduction pathways, as well as processing cytokines and chemokines, which upregulates immune response [14]. The resulting augmentation of inflammatory processes further induces the secretion of CCs, enhancing the destruction of ECM, which eventually leads to the acceleration of the processes observed in the aforementioned disorders.

Due to their roles in multiple inflammatory-based disorders, CCs have been considered for a long time as a target for medicinal drugs design. However, because of ubiquitous expression of most CCs, their constitutive, overlapping functions, broad substrate specificities, most of the studied so far medicines have exhibited unfavorable

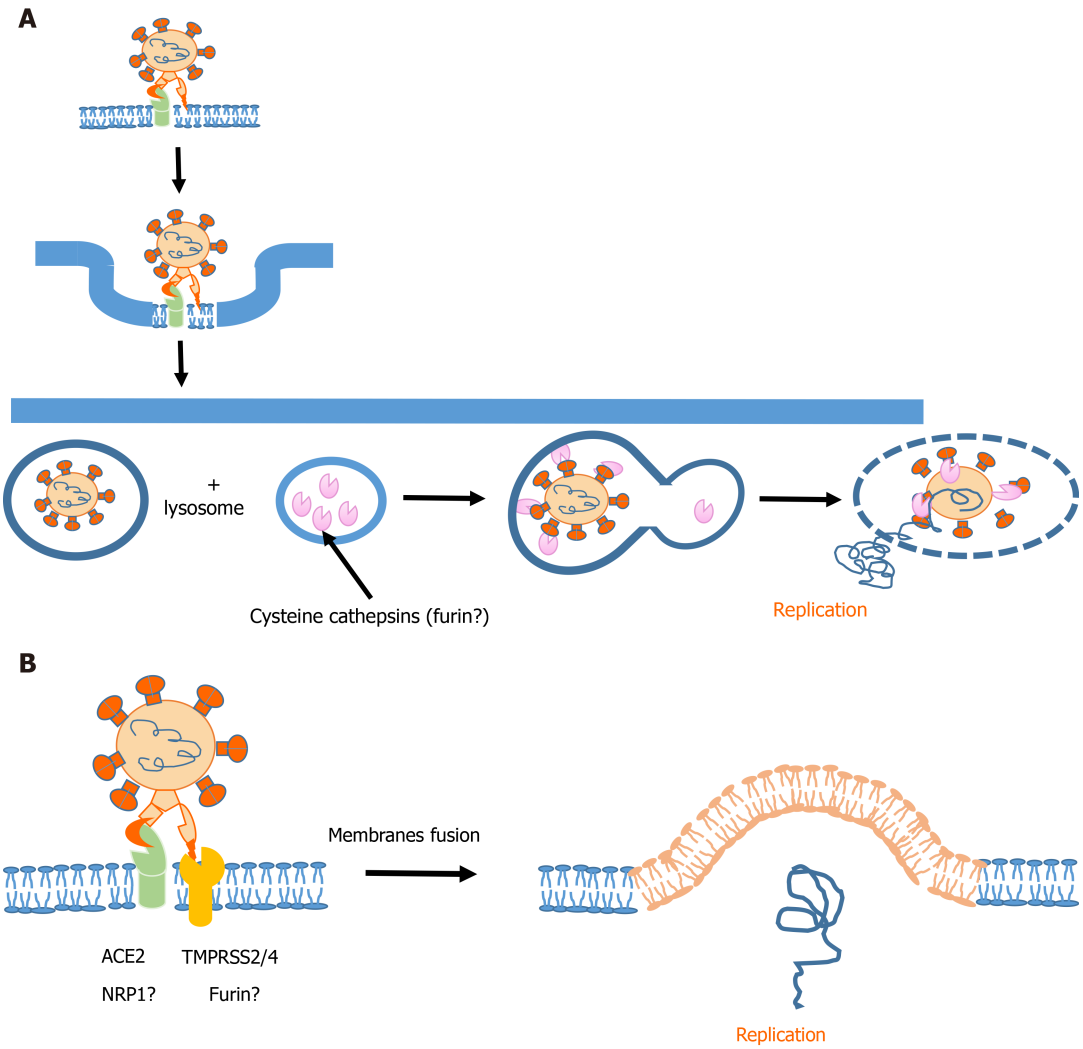

Figure 2 Two modes of virus entry. A: Receptor-mediated endocytosis of severe acute respiratory syndrome coronavirus-2. After binding to ACE2 and formation of endosome, lysosomal cathepsins activate spike protein, which leads to the release of the viral RNA into host cell. B: Membrane fusion mechanism - priming of the spike proteins is mediated by transmembrane peptidase/serine subfamily member 2/4, which leads to fusion of viral and host membranes and release of the viral RNA into host cell.

side effects, thus not surviving clinical trials. However, the attempts to construct CCs-aimed drugs, taking advantage of the newest technology, are underway. The most clinical trials have been conducted on cathepsins K and S inhibitors, also cathepsin C seems to be a promising target, whereas the remaining cathepsins inhibitors have either got stuck in the initial stages of clinical trials or their trials have been discontinued (reviewed in[14]).

Cathepsin L in inflammatory processes

Similarly to other cysteine cathepsins, cathepsin L exhibits pleiotropic activities in the human organism. One of the most evident actions of this enzyme is (beside cathepsins S, K and V) its participation in inflammatory processes associated with various pathological conditions[14-16]. For example Menzel *et al*[17] have demonstrated the up to 10-fold induction of cathepsin L expression (mRNA) in intestinal macrophages derived from inflammatory bowel disease (IBD) patients, and the clear improvement of the disorder symptoms in DSS (dextran-sulphate-sodium)-induced colitis mice mode, when a simultaneous application of cathepsins L and B inhibitors was investigated. Xu *et al*[18] have exhibited the stimulatory function of cathepsin L in microglia-mediated neuroinflammation, which accompanies many neurological disorders including Parkinson's disease. Cao *et al*[19] have shown the correlation between serum cathepsin L activity and the markers of inflammation (such as neutrophile counts and hs-CRP) in the patients with chronic kidney disease.

Cysteine cathepsins and TMPRSS2 in SARS-CoV-2 invasion

Cell line experimental systems creating the environment aimed at the inhibition of CCs have substantiated the function of these enzymes in the processes of SARS-CoV-2 activation. Raising pH in the endolysosomal compartments (with ammonium chloride and/or bafilomycin A), which inactivates lysosomal proteases working in acidic environment, or application of cysteine proteases inhibitors (such as E-64d inhibitor - inactivating cysteine cathepsins L, B, H, as well as cytosolic calpain) have significantly limited entry of SARS-CoV-2 into chosen cell lines[4]. Ou *et al*[20] (applying lentiviral pseudotype system) have demonstrated that the treatment of HEK-293/hACE2 cells with E-64d inhibitor reduced entry of SARS-CoV-2 S pseudovirions by over 90%. Further, the Authors compared the effect of two specific inhibitors of cathepsin L (SID 26681509), and cathepsin B (CA-074). Whereas the first inhibitor limited the pseudovirions entry by over 76%, the second one did not exhibit any significant effect, which suggests a prevalent function of cathepsin L in the receptor-mediated endocytosis mechanism of the virus invasion. RME mechanism in HEK- 293/hACE2 cells has been confirmed by the Authors in the experiments showing the inhibition of the SARS-CoV-2 S protein entry by blocking the factors inevitable in the process of endolysosomal trafficking (PI(3)P 5-kinase (PIKfyve) and two-pore channel subtype 2 (TPC2)[20]. The involvement of CCs has been also observed by Hoffmann *et al*[3] who demonstrated that the increase in pH (with ammonium chloride) almost completely inhibited SARS-2-S-driven entry into 293T cells expressing ACE2 but devoid of TMPRSS2. This observation is in agreement with Ou *et al*[20] findings confirming that the virus entry into these cells undergoes *via* RME with the involvement of cathepsin L in spike protein activation. In their experiments on the human colon cell line - Caco-2 cells overexpressing TMPRSS2, Hoffmann *et al*[3] exhibited the participation of both TMPRSS2 and CCs in the mechanism of the virus entry into these cells. Alkalization of the environment caused around 90% reduction in SARS-2-S-driven entry, whereas incubation with E-64d inhibited the virus entry by around 40%. On the other hand, the application of camostat mesylate (inhibitor of TMPRSS2 and other serine proteases) reduced the virus entry by about 90%, and when the Authors used both inhibitors simultaneously, they achieved nearly complete virus-entry inhibition to Caco-2 TMPRSS2 (+) cells. They also found that E-64 inhibitor significantly reduced the virus entry into Vero and 293T cells not expressing TMPRSS2, which indicates the endolysosomal pathway. However, the transduction of these cell lines with TMPRSS2 markedly reversed the effect of CCs inhibition, emphasizing the function of TMPRSS2 in the S protein priming, agreeably with Ou *et al*[20] findings that the expression of TMPRSS 2, 4, 11 A, 11D, and 11E on 293/hACE2 cells enhanced SARS-CoV-2 S protein-mediated cell–cell fusion. Moreover, the Authors noted that, however the addition of trypsin (like TMPRSS belonging to serine proteases) stimulated the formation of syncytia in 293/hACE2 cells (indicative of activated by trypsin SARS-2-S protein-stimulated cell-cell fusion), this process was also noticed in the experiments without trypsin. These findings indicate that binding with ACE2 receptors may be a sufficient event inducing cell-cell fusion (without the proteolytic priming with the extracellular protease). Nevertheless, it is possible that other host extracellular peptidases are involved in spike protein activation. The findings derived from Hoffmann *et al*[3] and Ou *et al*[20] experiments are depicted in Table 1.

Furin in SARS-CoV-2 invasion

As determined by Shang *et al*[8], another enzyme involved in SARS-CoV-2 spike protein priming is furin belonging to proprotein convertases (PCs). PCs are eukaryotic serine proteases, and ubiquitously expressed furin belongs to the PCs subfamily present in the organelles of the constitutive protein secretion pathway. These enzymes participate in the proteolytic post-translational modification of a variety of functionally important peptides and proteins, such as growth factors and hormones, both intra- and extracellularly (in the trans-Golgi network, endosomes, and pericellular environment). The amino acid sequence specifically recognized and cleaved by PCs including furin, is found in many viral surface proteins, so different viruses (like MERS-CoV) are activated by these enzymes[1]. Unlike SARS-CoV (exhibiting PC cleavage site motif only in the S2' site) [1], SARS-CoV-2 spike protein includes PCs specific motif at the S1/S2 boundary[8]. Shang *et al*[8] have performed experiments to examine whether this sequence is cut by furin. They demonstrated that PCs inhibitors reduced SARS-CoV-2 pseudovirus entry into three cell lines expressing hACE2 receptors; HeLa cells (human cervical cells), Calu-3 cells (human lung epithelial cells), and MRC-5 cells (human lung fibroblast cells). Moreover, the mutation of the PCs specific motif significantly reduced the pseudovirions entry to the studied cells. The

Table 1 Receptors/proteases involved in Severe acute respiratory syndrome coronavirus-2 invasion of human cells

Receptor/protease	Experimental model	Observation	Ref.
ACE2 and TMPRSS2/4	Human small intestinal enteroids; HEK-293T cell line transfected with ACE2, TMPRSS2, or TMPRSS4	Productive infection of SARS-CoV-2 in ACE2 (+) mature enterocytes; Correlation of ACE2, TMPRSS2 and 4 with SARS-CoV-2 invasiveness	Zang *et al*[11]
ACE2 and TMPRSS2	ACE2 and TMPRSS2 expressing C2BBe1, Caco-2, and Calu-3 cell lines	Persistent invasion and replication of SARS-CoV-2 in the cells; Correlation of TMPRSS2 (but not ACE2) with SARS-CoV-2 RNA	Lee *et al*[33]
ACE2, TMPRSS2, and CCs	HEK-293T cell line transfected with ACE2; Caco-2 cells overexpressing TMPRSS2	Inhibition of SARS-CoV-2 pseudovirus entry into HEK-293T ACE2(+)/TMPRSS2(-) by pH increase and E-64 inhibitor; Inhibition of SARS-CoV-2 pseudovirus entry into Caco-2 TMPRSS2(+) by pH increase, CCs inhibitor (E-64d), and TMPRSS2 inhibitor (camostat mesylate)	Hoffmann *et al*[3]
ACE2, TMPRSS 2, 4, 11A, 11D, 11E, and CCs (cathepsin L)	HEK-293T cell line transfected with ACE2	Inhibition of SARS-CoV-2 pseudovirus entry into the cells with CCs inhibitor (E-64d) and cathepsin L inhibitor (but not cathepsin B inhibitor); Intensification of SARS-CoV-2 S protein-mediated cell–cell fusion, caused by expression of TMPRSS 2, 4, 11A, 11D, and 11E on 293/hACE2 cells	Ou *et al*[20]
ACE2 and TMPRSS2	ACE2 and TMPRSS2 expressing Caco-2 and T84 cell lines	Persistent invasion and replication of SARS-CoV-2 in the cells	Stanifer *et al*[35]
ACE2, furin, TMPRSS2, and CCs	ACE2-expressing HeLa, Calu-3, and MRC-5 cell lines	Reduction of SARS-CoV-2 pseudovirus entry into cells by inhibitors of PCs, CCs and TMPRSS2	Shang *et al*[8]
NRP1, ACE2, and TMPRSS2	HEK-293T cell line transfected with ACE2, TMPRSS2, or NRP1	Augmentation of SARS-CoV-2 infectivity when NRP1 was coexpressed with ACE2 and TMPRSS2	Cantuti-Castelvetri *et al*[21]

ACE2: Angiotensin converting enzyme 2; NRP1: Neuropilin 1 (receptor which binds furin-cleaved substrates); TMPRSS2/4: Transmembrane peptidase/serine subfamily member 2/4; CCs: Cysteine cathepsins; HEK-293T: Human embryonic kidney 293T cells; Caco-2: Human colorectal adenocarcinoma cell line; C2BBe1: A subclone of Caco-2; T84: Human colon carcinoma cell line; HeLa: Human cervical cell line; Calu-3: Human lung epithelial cell line; MRC-5: Human lung fibroblast cell line; PCs: Proprotein convertases.

Authors detected no cleavage within the spike protein, when they packaged the pseudoviruses to HEK293T cells pretreated with furin-targeting siRNA. Additionally, they excluded the participation of matrix metalloproteinases (MMPs) in the experiment with the application of MMP inhibitor. Therefore, they confirmed the involvement of furin in SARS-CoV-2 entry into chosen cells. Additionally, furin priming of SARS-CoV-2 spike protein has been associated with the virions recognition and binding by neuropilin 1 (NRP1 - receptor which binds furin-cleaved substrates). Cantuti-Castelvetri *et al*[21] have shown that, except for ACE2, also NRP1 receptors are involved in SARS-CoV-2 invasion. Although the exact mechanism of NRP1 participation in this process is not elucidated, the Authors found the receptors to markedly enhance the virus infectivity. Apart from furin involvement, Shang *et al*[8] also observed the association of other aforementioned peptidases with SARS-CoV-2 invasion. They noticed the reduction in the pseudovirus entry to the three studied cell lines, caused by the application of both serine proteases (camostat) and cysteine cathepsins (E64) inhibitors. Furthermore, the pseudovirus entry to HeLa cells was more markedly reduced when either camostat or E64d was applied in the cells pretreated with proprotein convertases inhibitor[8]. Therefore, it might be concluded that depending on the type of target cells, TMPRSS2, lysosomal cathepsins, and furin might be involved in the activation of SARS-CoV-2 entry exhibiting cumulative/overlapping final effect (Figure 2). The observations of Shang *et al*[8] and Cantuti-Castelvetri *et al*[21] are collected in Table 1.

Cysteine cathepsins as a target in search for COVID-19 therapy

Focusing on cathepsin L expression as a target in search for an anti-Covid-19 drug, Smieszek *et al*[22] have tested an array of medications applied in clinical practice. They found amantadine (a drug used previously to treat influenza A, and now applied in neurological diseases including Parkinson's disease) to be a promising compound. Being able to accumulate in lysosomes and alkalize them, amantadine belongs to lysosomotropic agents. Such compounds inactivate lysosomal enzymes including CCs whose optimal pH lies below 5. The Authors demonstrated a significant reduction in cathepsin L gene expression using amantadine, however also other cysteine cathepsin

genes expression was inhibited, including cathepsins B and K, with the most pronounced effect observed for cathepsin H. Therefore, it might be hypothesized that this is also cathepsin H which plays an important role in the processing of the SARS-2-S spike protein. The activity of cathepsin H would have been inhibited similarly to other lysosomal cathepsins in the aforementioned experiments (raising pH and/or using E64 inhibitor), which demonstrated a significant reduction of SARS-2-S-mediated entry to the studied cells. However, in comparison with thoroughly studied cathepsins B, L, S, and K, there is much less scientific data referring to cathepsin H.

Amantadine efficiency in COVID-19 treatment has been suggested by Rejdak *et al* [23] who documented no clinical manifestations of COVID-19 infection in 22 patients in spite of the confirmation of SARS-CoV-2 presence with rRT-PCR testing in all of these individuals. The patients had been treated with either amantadine or memantine, for at least 3 months prior to the infection exposure, due to their conditions (multiple sclerosis, Parkinson's disease or cognitive impairment). Therefore, amantadine seems to be a promising treatment for COVID-19 patients. The effect of amantadine may be associated with the down-regulation of cysteine cathepsins (L and/or H) in the endolysosomal compartment and/or the disturbance of viroporin protein channel probably involved in the viral RNA release, as suggested for SARS-CoV[24].

As discussed before, the generation of anti-CCs medicinal drugs is a problematic issue (associated with the observation of unfavorable side effects). Hence, the attitude aimed at screening the already existing therapeutics, which would lower the activity of proteases involved in SARS-CoV-2 activation, seems a rational approach.

EFFECT OF SARS-CoV-2 ON GASTROINTESTINAL TRACT

Gastrointestinal symptoms and fecal virus shedding in COVID-19 patients

A significant amount of scientific evidence accumulated so far points to gastrointestinal tract (GIT), especially its lower part, as a target organ affected by SARS-CoV-2, beside the respiratory system[25]. Apart from the typical pulmonary symptoms (cough, fever, shortness of breath), some of the patients (around 4%-50% individuals) present with digestive symptoms like diarrhea, nausea, vomiting and abdominal pain[26]. Moreover, the virus mRNA presence in stool samples has been observed in some patients, often persisting long after its disappearance from the respiratory tract. Wu *et al*[27] have documented SARS-CoV-2 mRNA presence in the feces of more than half of the studied patients, with duration for up to 5 wk after its vanishing from the respiratory tract specimens. It might suggest active proliferation of the virus in the gastrointestinal tract of some patients. Similarly, Xiao *et al*[28] have observed fecal virus shedding in more than 50% of the studied patients, and in over 20% of them the duration of positive results in stool exceeded the virus presence in the respiratory samples. Additionally, the Authors detected the protein parts of the virus (as well as the presence of ACE2 receptors) in gastrointestinal epithelial cells. The virus replication in rectal tissue derived from a COVID-19 patient has been noted by Qian *et al*[29] who detected SARS-CoV-2 components in the intestinal epithelial cells (but mainly in intestinal lymphocytes and macrophages). The Authors hypothesize that, like in the case of influenza virus, it is possible for SARS-CoV-2 virions to be transported from the respiratory tract to the GIT *via* the immune cells. In the meta-analysis of 60 studies comprising over 4000 COVID-19 patients, performed by Cheung *et al*[30], 17.6% of the patients exhibited gastrointestinal symptoms, and in almost 50% (30%-70%) the virus mRNA was detected in the feces. Most of these positive stool samples (above 70%) were collected after the loss of the virus from the respiratory specimens.

However, it is still not clear, whether the virus is transmittable *via* fecal-oral route. Whereas Wang *et al*[31] detected live virus in the fecal samples, Zang *et al*[11] demonstrated the inactivation of the virions released into the intestinal lumen in the environment simulating human colonic fluid. Moreover, the Authors did not manage to recover infectious virus from the stool specimens.

Ability of SARS-CoV-2 to productively infect human GIT cells via ACE2 and TMPRSS

Several studies have demonstrated the invasion and replication of SARS-CoV-2 in the human GIT cells. Chu *et al*[32] in their ex-vivo experiments on human intestinal tissues have evidenced the ability of SARS-CoV-2 to infect, proliferate and release infectious virus particles from intestinal cells. In comparison with SARS-CoV, SARS-CoV-2 replicated less efficiently and brought about less damages in the human intestinal

epithelium, but evoked greater response of innate immune system (inducing the expression of proinflammatory mediators such as interferons and interleukins). Hence, the Authors suggested that the gastrointestinal tract might serve as an alternative route of virus dissemination. The vulnerability of the human GIT epithelial cells to SARS-CoV-2 invasion *via* ACE2 and TMPRSS-2 receptors has been confirmed in other studies with the application of intestinal cell lines models, as well as human small intestinal organoids – hSIOs[11,33-35]. Lee *et al*[33] investigated the growth of SARS-CoV-2 in a human GIT cell line model; C2BBe1 (a subclone of human epithelial colorectal adenocarcinoma cells: Caco-2). C2BBe1 (genetically and structurally resembling the brush border epithelial cells in the human GIT[36,37], and expressing moderate level of ACE2 and high level of TMPRSS2[33]) exhibited the greatest susceptibility to the virus. SARS-CoV-2 virions invaded and replicated in these cells, as well as in Caco-2[33,35] and T84 (human colon carcinoma) cells[35]. Furthermore, Stanifer *et al*[35] demonstrated SARS-CoV-2 infection of human colon organoids followed by active virions replication. These observations are in agreement with other studies on hSIOs[11,34]. Zang *et al*[11] reported productive infection of SARS-CoV-2 in $ACE2^{(+)}$ mature enterocytes, dependent on TMPRSS2 and TMPRSS4 receptors in human small intestinal enteroids. The Authors noted the role of an additional serine protease: TMPRSS4 which heightened the effect of TMPRSS2. Also, the two serine proteases enhanced SARS-CoV-2 spike protein-induced cell-cell fusion observed by the Authors.

ACE2 receptors (except for the respiratory system) are present in a variety of other organs including the gastrointestinal tract, where a great number of these proteins has been detected in the lower part of GIT[38]. Unlike in the upper segment (oral cavity, esophagus, stomach), high expression of ACE2 (both mRNA and protein) is observed in the small intestine (the greatest level), colon and rectum, as well as in the gall bladder[38,39]. Actually, ACE2 expression in the small intestine is much higher in comparison with all other organs in the human organism including the respiratory tract[11]. Specifically, ACE2 is present in the enterocyte cytoplasm and in the apical brush border, as well as in the glandular cells (in the lining epithelium of the lower GIT)[37]. The expression of ACE2 receptors has been exhibited to increase upon enterocytes differentiation. Lee *et al*[33] observed that (unlike constitutively expressed TMPRSS2), the expression of ACE2 receptors was significantly stimulated in the experiment inducing C2BBe1 enterocytes differentiation, associated with the generation of more pronounced features typical for brush border cells. Similarly, Zang *et al*[11] detected the greatest expression of ACE2 in mature brush border enterocytes, and Lamers *et al*[34] noticed around 1000-fold increase in ACE2 mRNA expression upon enterocytes differentiation. Zang *et al*[11] observed all studied receptors (ACE2, as well as TMPRSS2 and TMPRSS4) to be correlated with the virus invasiveness, similarly as Lee *et al*[33] noted a strong correlation between TMPRSS2 (although not ACE2) and viral RNA levels in the studied human epithelial cell lines (including Caco-2 and C2BBe1). Lee *et al*[33] found that the ectopic coexpression of ACE2 and TMPRSS2 in RPMI 2650 cells enhanced viral dissemination by 56.7 times (over 10-fold more in comparison with the sole ACE2 effect – 4.9 times, whereas TMPRSS2 transfection alone did not enhance the level of infectivity). It might be supposed that an effective level of ACE2 receptors is a prerequisite for the virions invasion of epithelial cells, but abundant expression of TMPRSS2 (and possibly TMPRSS4) greatly facilitates ACE2-mediated SARS-CoV-2 dissemination in the human GIT.

The involvement of both ACE2 and TMPRSS2 in SARS-CoV-2 invasion of GIT epithelial cells is in accordance with the reported co-localization of the two receptors in the lower GIT[38-40]. The most abundant expression of both proteins has been detected in the small intestine epithelial cells[38]; especially in the brush border cells [33]. Lee *et al*[39] evaluated single-cell RNA-sequencing datasets from the GIT in search for these two genes co-expression, and found the small intestine enterocytes as well as colonocytes to display the highest proportions of cells co-expressing ACE2 and TMPRSS2. Additionally, the Authors checked for the co-expression of ACE2, TMPRSS2 and TMPRSS4, and demonstrated the highest proportions of the three genes co-expression in the progenitor and stem-like epithelial cells in the small intestine. TMPRSS4 is an extra serine protease involved in SARS-CoV-2 activation and invasion, enhancing TMPRSS2 priming effect[11]. Therefore, both ACE2 and TMPRSS2 seem to be the main receptors responsible for SARS-CoV-2 invasion of GIT. The experimental data coming from Lee *et al*[33], Zang *et al*[11], and Stanifer *et al*[35] investigations are displayed in Table 1.

Putative association between SARS-CoV-2-mediated ACE2 disturbance and gastrointestinal symptoms development in COVID-19 patients

The known function of angiotensin converting enzyme 2 (ACE2) is the regulation of systemic arterial blood pressure (renin-angiotensin system). ACE2 catalyzes the conversion of Ang I to angiotensin (1–9) and angiotensin II (Ang II) to angiotensin (1–7), what counteracts the effects of Ang II, leading to decrease in blood pressure and inflammatory processes attenuation (reviewed in[41]). However, the role of ACE2 in GIT (where it is abundantly expressed) seems to be rather associated with the processes occurring in this organ. The analysis of the digestive system specific functional enrichment map for ACE2 gene suggests the involvement of ACE2 in digestion (with reference to its proteolytic activity) and transport of metabolites (the regulation of amino acid transport)[38]. In fact, ACE-2 proteins have been demonstrated to be coupled with sodium-dependent amino acids and glucose transporters. ACE2 is a chaperone for the sodium-dependent amino acid transporter B0AT1 which is involved in transport of neutral amino acids[42]. Moreover, ACE2 participates in the regulation of gut microbiota homeostasis[43,44].

Therefore, as proposed by Kumar *et al*[38], it might be hypothesized that SARS-CoV-2-associated dysregulation of ACE2 receptors in the human GIT may be involved in the mechanism of GIT symptoms development in COVID-19 patients.

CONCLUSION

In light of the presented studies, the impact of SARS-CoV-2 virus on GIT is evident, with the most substantiated involvement of ACE2 and TMPRSS2. However, except for these two receptors (and probably TMPRSS4), also other proteases might be implicated in SARS-CoV-2 invasion of GIT, as well as the development of the observed symptoms. Ubiquitously expressed furin and cathepsin L may be involved in spike protein processing, contributing to the virus invasiveness. Cathepsin L (and other CCs) might participate in the endolysosomal processing of the spike protein following ACE2-mediated endocytosis of the virion particles. Additionally, SARS-CoV-2 – induced inflammatory cytokines could stimulate the secretion of cathepsin L. Therefore, extracellular cathepsin L may contribute both to the spike protein processing, as well as degradation/remodeling of the ECM components and membrane-bound receptors including TMPRSS2/4 and ACE2. The resulting events might accelerate the inflammatory processes disturbing the digestion/absorption of nutrients yielding the observed symptoms such as diarrhea. However, more research is required, since the participation of furin and lysosomal cathepsins in SARS-CoV-2 GIT-invasion is more speculative.

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MINIREVIEWS

Endoscopic anti-reflux therapy for gastroesophageal reflux disease

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Abstract

Gastroesophageal reflux disease has an increasing incidence and prevalence worldwide. A significant proportion of patients have a suboptimal response to proton pump inhibitors or are unwilling to take lifelong medication due to concerns about long-term adverse effects. Endoscopic anti-reflux therapies offer a minimally invasive option for patients unwilling to undergo surgical treatment or take lifelong medication. The best candidates are those with a good response to proton pump inhibitors and without a significant sliding hiatal hernia. Transoral incisionless fundoplication and nonablative radiofrequency are the techniques with the largest body of evidence and that have been tested in several randomized clinical trials. Band-assisted ligation techniques, anti-reflux mucosectomy, anti-reflux mucosal ablation, and new plication devices have yielded promising results in recent noncontrolled studies. Nonetheless, the role of endoscopic procedures remains controversial due to limited long-term and comparative data, and no consensus exists in current clinical guidelines. This review provides an updated summary focused on the patient selection, technical details, clinical success, and safety of current and future endoscopic anti-reflux techniques.

Key Words: Treatment; Gastroesophageal reflux; Transoral incisionless fundoplication; Anti-reflux mucosectomy; Anti-reflux mucosal ablation; Stretta

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Core Tip: Gastroesophageal reflux disease is a common disorder that impacts quality of life. Endoscopic anti-reflux therapies are intended to offer an alternative for patients unwilling to undergo surgical treatment or take lifelong medication. Several techniques, such as transoral incisionless fundoplication, nonablative radiofrequency, plication methods, and anti-reflux mucosectomy, have shown encouraging results, but their role in the management of gastroesophageal reflux disease remains controversial. Careful patient selection and awareness of the advantages and disadvantages of each technique are essential to optimize outcomes. We herein provide an updated review of the technical aspects, clinical success, and safety of the principle endoscopic anti-reflux procedures.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a condition that develops when reflux of stomach contents causes troublesome symptoms or complications in the esophagus or beyond[1,2]. GERD is very frequent worldwide, with a prevalence ranging from 7.4% in Southern Asia to 19.6% in Central America, and it affects both sexes similarly[3]. The increment in aging and obesity, both predisposing factors for GERD, may increase its impact in the near future even further[4]. Many other factors also favor GERD exacerbation, including tobacco and certain drugs, such as calcium blockers and tricyclic antidepressants[5,6]. GERD negatively affects quality of life and imposes economic and productivity loss burdens[7].

Although the cause of GERD is still incompletely understood, several underlying predisposing pathophysiological mechanisms have been described. While low esophageal sphincter (LES) basal pressure may facilitate reflux after abdominal strain or during swallowing, a more pertinent mechanism is transient LES relaxation (TLESR), which can be associated with esophageal shortening[8,9]. Gastroesophageal junction (GEJ) disruption due to a hiatal hernia (HH) constitutes an additional factor because it contributes to LES incompetence and also displaces the acid pocket closer to the esophageal mucosa[10,11]. Altered visceral sensitivity has a bidirectional effect in GERD, magnifying symptoms in patients without mucosal injury and reducing symptom awareness in Barrett's esophagus patients[12]. Esophageal hypomotility, low saliva production, and other mechanisms such as certain breathing patterns may also contribute to GERD[13].

The management of GERD is multimodal. Lifestyle modifications such as weight loss, tobacco cessation, and, in selected cases, postural advice[14] have proven efficacy and may be sufficient in mild cases. Drug therapy occupies the next level, with proton pump inhibitors (PPIs) having a huge impact on GERD treatment due to high esophagitis healing rates, surpassing the performance of histamine receptor antagonists and exhibiting high cost-efficacy[15,16]. They are the cornerstone of medical GERD treatment. Anti-reflux surgery (ARS), namely laparoscopic fundoplication, is the last step in GERD management. Its objectives are as follows: (1) LES fixation to the hiatus and intraabdominal segment length augmentation; (2) LES basal pressure increase; and (3) hiatal repair. The latter aspect appears crucial because hiatal repair itself impacts the length and pressure of the LES more than fundoplication[17]. Randomized controlled trials (RCTs) have failed to demonstrate a clear long-term superiority of ARS over PPIs[18]. Consequently, ARS is reserved for patients who do not respond to PPIs, do not tolerate them due to adverse effects, or are unwilling to maintain them in the long term.

PPI refractoriness probably constitutes the most frequent indication for surgery, although it is a confusing term and thus deserves further consideration. The same concept frequently encompasses vastly different realities. Refractoriness can be partial or complete, a distinction that is clinically relevant. Recent and major trials have defined the grade of refractoriness needed to meet inclusion criteria[19]. Subsequently,

symptoms can persist for very different reasons, such as poor adherence to medical therapies, absence of a relationship with reflux (*e.g.*, functional heartburn), or objectively proven reflux persistence despite proper medical treatment. Therefore, guidelines advise a full diagnostic workup before surgery to demonstrate as consistently as possible that the symptoms, whether refractory or not, are objectively secondary to GERD[2,20-27].

In the last 30 years, effort has been made to design endoscopic anti-reflux therapies that serve as a valuable option for GERD management, either as an alternative to ARS or as bridge therapy between pharmacological treatment and surgery. They do not thus far allow hiatal repair and constrain candidate selection to individuals without a HH. In 1979, Angelchik[28] used a silicon prosthesis as the first endoscopic treatment for GERD. Since then, numerous other treatments have emerged, with many, such as GEJ injections of bulking agents and several plication techniques, disappearing because of low efficacy or unacceptable adverse effects[29-31]. Here, we present a comprehensive review of the endoscopic approaches for the treatment of GERD that have survived the test of time or have recently been designed (Table 1).

INDICATIONS FOR ENDOSCOPIC ANTI-REFLUX THERAPY

Endoscopic therapies should be considered at least in the same scenarios as surgery and should offer some advantages over ARS. Specifically, endoscopic anti-reflux therapy should be considered in PPI nonresponders, in patients who have a contraindication to PPIs or have concerns regarding their long-term adverse effects, and in those who either do not qualify for ARS or refuse it. Ideally, endoscopic techniques should demonstrate noninferior efficacy, alongside a shorter operation time, lower complication rate, and lower secondary long-term morbidity. Finally, they should not preclude a future fundoplication in case of failure.

Laparoscopic fundoplication performed by skilled surgeons has a low short-term morbidity and mortality but can cause significant adverse effects in the medium term, such as dysphagia (in up to 24% of patients), gas-bloat syndrome, and incisional hernia, and revision surgeries are not infrequent[22]. It fails in 10%-15% of patients in the short term, and long-term studies have shown that more than 30% of patients are still on PPIs years after surgery[22,32]. This constitutes the scenario against which endoscopic therapies should be compared.

The guidelines of the main medical and surgical societies and expert consensus documents published in the last 10 years have addressed the endoscopic alternatives as well as the surgical option. Their recommendations and the level of evidence or consensus that they are based upon are summarized in Table 2[2,20-26,33-35]. Transoral incisionless fundoplication (TIF) and nonablative radiofrequency are considered appropriate in well-selected patients and situations according to recent guidelines.

CURRENT ENDOSCOPIC THERAPIES

Transoral incisionless fundoplication

The aim of TIF is to perform an endoscopic fundoplication by reestablishing the flap valve mechanism with a 3-cm high-pressure zone at the distal esophagus to durably restore LES function[36]. This procedure mirrors ARS by using an endoscopic suturing device with T-fasteners, the EsophyXâ device (EndoGastric Solutions, Inc., Redmond, WA, United States)[37]. These devices have evolved from a longitudinally oriented gastrogastric plication to one with a greater degree of rotational movement, 200° to 300° in circumference and a 2-3-cm length wrap over the distal esophagus below the diaphragm to create full-thickness serosa-to-serosa esophagogastric plications. This easier to use and more automated device can deploy about 20 fasteners without the need for visualization of the stylet/fastener deployment. The objective of the technique is to restore the integrity of the angle of His by firing stabilizing T-fasteners, deployed 2 to 3 cm above the GEJ, with a 270° esophagogastric wrap, to mimic a Toupet surgical fundoplication. The EsophyXâ device was approved in 2007 by the United States Food and Drug Administration as a single-use, two-operator device comprising a tip (tissue retractor, tissue mold and chassis, fasteners over a stylet, and the invaginator) and body (H-fasteners, helix retractor lock, vacuum connection, fastener pusher, helix retractor control, tissue mold knob, gastroscope point of insertion).

Table 1 Comparison of current endoscopic therapies for gastroesophageal reflux disease

	TIF	MUSE	Stretta®	GERDx™	ARMS/ARMA	Band ligation
Efficacy	++	+	+ -	+	+	+
Safety	+	+	++	+	+	+
Technical difficulty	++	++	+	++	+	+
Add-on device	+	+	+	+	-	-
RCT available	+	-	+	-	-	-
Maximum follow-up (yr)	10	5	10	0.25	3	1
Cost	++	++	++	++	+	+

++: Indicates the highest score; +: Indicates a moderate score or yes; -: Indicates uncertainty; TIF: Transoral incisionless fundoplication; MUSE: Medigus ultrasonic surgical endostapler; GERDx™: Endoscopic full-thickness plication device; ARMS: Anti-reflux mucosectomy; ARMA: Anti-reflux mucosal ablation; RCT: Randomized controlled trial.

Optimal candidates for TIF are patients who demonstrate LES incompetence (Hill grade II) without a concomitant HH. TIF 1.0 has been discontinued because TIF 2.0 achieves much better results[36]. The improved procedure has been evaluated in nine noncomparative studies[38-46] and in five RCTs[47-51] comprising 886 patients with moderate GERD without a large HH, Los Angeles grade C or D esophagitis, or Barrett´s esophagus (Table 3). Clinical success rates ranged from a modest 50% at 12 mo to as high as 92% at 10 years. Severe adverse events (SAEs) have been reported in 2.4% of patients[52]. A recent network meta-analysis suggested that the TIF 2.0 procedure manages symptoms and allows PPI discontinuation at rates similar to those of ARS with an improved safety profile and fewer long-term adverse events[53]. A clinical response, defined by an improvement of at least 50% in GERD health-related quality of life (GERD-HRQL) score or remission of heartburn and regurgitation, was observed in 66% of patients treated with TIF. Moreover, TIF had the highest probability of improving GERD-HRQL (0.96), followed by ARS (0.66) and PPIs (0.042). In contrast, ARS had the highest probability of increasing the percent time at pH < 4 (0.99), followed by PPIs (0.64) and TIF (0.32)[53]. A review of the published evidence supports the belief that most selected patients undergoing TIF 2.0 experience a long-term elimination of GERD symptoms with no SAEs and that this procedure is a cost-effective alternative to ARS.

Medigus ultrasonic surgical endostapler

The Medigus ultrasonic surgical endostapler (MUSE), or MUSE™ system (Medigus, Omer, Israel), combines microvisual, ultrasonic, and surgical stapling capabilities into one device, which enables a single endoscopist to perform a transoral anterior fundoplication. This flexible surgical endostapler resembles an endoscope with a rigid section holding a cartridge with five standard 4.8-mm titanium surgical staples. The distal tip contains an anvil for bending the staples, two small 21-gauge screws, and an ultrasonic transducer to measure the distance to the cartridge. This method is a three-step procedure: (1) The stapler is advanced into the stomach through an overtube and retroflex; (2) The system is retracted to 3 cm proximal to the GEJ for clamping when the tissue thickness is 1.4-1.6 mm, and the stapler is then fired; and (3) The procedure is repeated to add quintuplets of staples to create an anti-reflux barrier.

This endoscopic stapling system has been evaluated in four noncomparative studies [46,54-56] and in one two-arm case series study[57] including 209 patients with GERD without a HH larger than 3 cm (Table 3). Clinical success rates ranged from 69% to 92% with follow-up durations from 6 mo to 5 years. The risk of SAEs (empyema, hemorrhage, esophageal perforation) was 3.5%. Overall, data on the efficacy and safety of MUSE are scarce and evidence from RCTs is lacking.

Nonablative radiofrequency treatment (Stretta®)

This endoscopic-guided method involves the application of radiofrequency energy to the muscle fibers of the LES and the gastric cardia, through the Stretta® system (Restech, Houston, TX, United States). The Stretta® catheter is introduced over the guidewire and positioned sequentially at three levels: 0.5 cm proximal to the GEJ, at the GEJ, and 0.5 cm below the GEJ. At each level, the balloon basket assembly is

Table 2 Summary of guidelines and consensus recommendations and invasive gastroesophageal reflux disease therapies

Society guidelines and year of publication	Indication for surgery	Strength of recommendation, level of evidence, and grade of consensus	Endoscopic anti-reflux therapy addressed	Guideline recommendation on endoscopic anti-reflux therapy	Strength of recommendation and level of evidence
ACG guidelines for diagnosis and management of GERD, 2013[2]	Option for long-term treatment	Quality: High. Strength: Strong	Radiofrequency, bulking agents, endoscopic suturing	Not recommended	Quality: Moderate. Strength: Conditional
	Generally not recommended in PPI-unresponsive patients	Quality: High. Strength: Strong			
	Refractory patients with objective evidence of ongoing reflux as the cause of symptoms	Quality: Low. Strength: Conditional			
EAES recommendations, 2014[22]	Good response but dependent on long-term PPI therapy, after optimal risk-benefit discussion	Grade: C. Consensus: 100%	Radiofrequency (Stretta®), bulking agent injection (Enteryx®), plication (EndoCinch®, full-thickness plication, EsophyX®	Not enough evidence available to recommend any as an alternative option to surgery	Grade of recommendation: B. Expert consensus: 100%
	Total or partial refractoriness despite adequate PPI therapy in terms of dosage and intake	Grade: A. Consensus: 100%			
	Well-selected NERD patients and those with hypersensitive esophagus	Grade: C. Consensus: 100%			
American Society of Gastrointestinal Endoscopy: The role of endoscopy in the management of GERD, 2015[95]	Not provided	Not provided	Radiofrequency (Stretta®) and transoral incisionless fundoplication	Consider in highly selected patients. No details on selection criteria	Low quality
Asia-Pacific consensus on refractory GERD management, 2016[23]	Refractory symptoms with objectively documented GERD	Quality: Moderate. Strength: Strong. Consensus: 100%	None	Not applicable	Not applicable
World Gastroenterology Organisation Global Guidelines, 2017[24]	Large hiatal hernia with volume-related reflux symptoms. Refractory esophagitis. Refractory symptoms documented as caused by GERD. Medication adverse effects	Not specified	Endoscopic therapies in general	Only in the context of clinical trials	Not specified
SAGES Guidelines on GERD surgical treatment, 2010, and on endoluminal anti-reflux treatments, 2017[21,34]	Appropriately selected GERD patients	Grade A	Transoral incisionless fundoplication	Control of symptoms in appropriately selected patients in the short term; appears to lose effectiveness	Quality: Moderate. Strength: Strong
			Radiofrequency	Control of symptoms in appropriately selected patients; long-term effect in appropriately selected patients	Quality: Moderate. Strength: Strong
USA expert panel (surgeons and advanced therapeutic endoscopists) recommendations on GERD management, 2020[25]	PPI responders (complete or partial)	Appropriate. Consensus: 87%-100%	Transoral incisionless fundoplication	PPI responders (complete or partial), no hernia, any other scenario	Appropriate. Consensus: 93%
				PPI responders (complete or partial) or nonresponders, significant hernia, any other scenario	Not appropriate
	PPI nonresponder, no hernia, heartburn-hypersensitivity, or negative pH-impedance	Appropriateness uncertain		PPI nonresponder, no hernia and acid breakthrough, hypersensitivity or	Appropriate. Consensus: 80%–93%

	study			negative pH-impedance study for heartburn	
	PPI nonresponder, any other scenario	Appropriate. Consensus: 80%-100%		PPI nonresponder, regurgitation, negative pH-impedance study	Appropriateness uncertain
			Radiofrequency	PPI responders (complete or partial) or nonresponders, no hernia, any scenario	Appropriateness uncertain
				PPI responders (complete or partial) or nonresponders, significant hernia	Not appropriate
ESGE guidelines on endoscopic management of gastrointestinal motility disorders, 2020 [35]	Not applicable	Not applicable	Transoral incisionless fundoplication	Possible role in mild GERD patients who are unwilling to take PPIs or undergo surgery. Against widespread use	Quality: Moderate. Strength: Strong. Consensus: 92.8%
			Medigus Ultrasonic Surgical Endostapler	Insufficient data. Use only in clinical trials	Quality: Low. Strength: Strong. Consensus: 100%
			Radiofrequency	Can be considered in selected patients only, without erosive esophagitis and hiatal hernia	Quality: Moderate. Strength: Weak. Consensus: 92.9%
			Anti-reflux mucosectomy	Against routine use in clinical practice	Quality: Low. Strength: Strong. Consensus: 100%
ESNM/ASNM consensus paper on management of refractory GERD, 2020 [26]	Refractory GERD symptoms in patients with proven GERD	Consensus: 100%	Transoral incisionless fundoplication	Short-term benefit in improving regurgitation in carefully selected patients	Consensus: 100%
			Radiofrequency	Variable symptom improvement, limited objective improvement in acid burden or manometric esophagogastric junction features	Consensus: 100%

ACG: American College of Gastroenterology; EAES: European Association of Endoscopic Surgery; SAGES: Society of the Americans Gastrointestinal and Endoscopic Surgeons; GERD: Gastroesophageal reflux disease; ESGE: European Society of Gastrointestinal Endoscopy; ESNM: European Society of Neurogastroenterology and Motility; ASNM: American Society of Neurogastroenterology and Motility; PPIs: Proton pump inhibitors; NERD: Nonerosive reflux disease.

inflated and then four nitinol needle electrodes (22-gauge, 5.5-mm) are extended into the muscular layer to deliver a radiofrequency current and induce a thermal reaction. Next, to deliver radiofrequency energy to four additional points, the catheter is rotated 45º clockwise[58]. The pathophysiological mechanism is not fully understood, but the thermal injury is thought to promote submucosal fibrosis and muscularis propria hypertrophy, which would decrease the frequency of TLESR and GEJ compliance while increasing LES and gastric yield pressures[58].

The Stretta® procedure has been evaluated in numerous cohort studies and in five RCTs, three with sham therapy and two with PPI use[59] (Tables 1 and 3). The RCT results did not show significant changes in esophageal acid exposure at 6 mo following Stretta®, compared with the PPI group[60]. Likewise, patients treated with Stretta® presented significant improvements in heartburn symptoms and quality of life in only the short term, compared with a sham procedure, with no long-term data[61-63]. A meta-analysis including 159 patients, limited to four RCTs, confirmed the absence of significant changes in patients with GERD[64]. More recently, a second meta-analysis that included both RCTs and 24 other cohort studies with 2468 evaluated patients[65] showed a significant postprocedural improvement in quality of life and in heartburn score but no improvement in basal LES pressure. The procedure is safe and well-tolerated, and SAEs are very rare. RCTs and cohort studies reported erosions, mucosal lacerations, gastroparesis, mediastinal inflammation, pneumonia,

Table 3 Clinical success and safety of endoscopic therapies

Technique	Study design and population	Clinical success, range	Major adverse events, range
Transoral incisionless fundoplication	No. of RCTs: 5; *n* = 343 No. of nonrandomized case series: 9; *n* = 543	50%–92%	0%–4.4%
Medigus ultrasonic surgical endostapler	No. of RCTs: 0 No. of nonrandomized case series: 5; *n* = 199	69%–92%	0%–9%
Nonablative radiofrequency (Stretta®)	No. of RCTs: 5; *n* = 173 No. of nonrandomized case series: 29; *n* = 2571	15%–100%	0%–1%
Endoscopic plication device (GERDx™)	No. of RCTs: 0 No. of nonrandomized case series: 1; *n* = 40	19 out of 40 patients were off PPIs	10%
Band ligation techniques	No. of RCTs: 1; *n* = 150 No. of nonrandomized case series: 2; *n* = 73	43%–54%[1]	0%
Anti-reflux mucosectomy	No. of RCTs: 0 No. of nonrandomized case series: 12; *n* = 331	58%–100%	0%–17%
Anti-reflux mucosal ablation	No. of RCTs: 0 No. of nonrandomized case series: 3; *n* = 130	58%–89%	0%–13%

[1]Clinical success not defined in the randomized controlled trial. There was a significant reduction in gastroesophageal reflux disease health-related quality of life score and 24-h pH-metry outcomes. RCT: Randomized controlled trial; PPIs: Proton pump inhibitors.

and pleural effusion[66].

Endoscopic plication device (GERDx™)

The GERDx™ device (G-SURG GmbH, Seeon-Seebruck, Germany) uses hydraulic elements for control and requires a slim gastroscope that works as a light source. It is the advanced single-use product of the company that has acquired the Plicator technology after withdrawal of the Plicator device (Ethicon Endo-Surgery, Sommerville, NJ) from the market. The experience with GERDx™ is still minimal, with only two publications in this regard, one of which is an interim analysis by the same authors (Tables 1 and 3).

In a single-center, single-arm trial, Weitzendorfer *et al*[67,68] prospectively assessed the outcomes of 40 patients with refractory GERD treated with the GERDx™ device. Of the 40 patients, 7 underwent LARS before the 3-mo follow-up. The mean DeMeester score was reduced from 46.48 to 20.03 in the 30 patients who completed the follow-up. Of these 30 patients, 18 (60.0%) achieved normal DeMeester score levels. In addition, 3 (10.0%) stated that they were on daily PPI medication after the plication, with 8 (26.7%) taking on-demand medication and 19 (63.3%) off medication. Moderate SAEs were reported by 10% of the patients (a hematoma at the GEJ, a case of pneumonia, a suture passing through the left hepatic lobe, pleural empyema, a severe Mallory-Weiss tear). The single-study evidence, lack of a comparator arm, and the very short follow-up make this endoscopic treatment experimental at this time, necessitating new RCTs to corroborate improvements in quality of life and acid exposure and confirm procedural safety.

Anti-reflux mucosectomy and anti-reflux mucosal ablation

Anti-reflux mucosectomy (ARMS) was first devised in a patient with a Barrett's esophagus-related lesion treated by endoscopic submucosal dissection. The resulting scar improved GERD symptoms and normalized the DeMeester score[69]. This observation led to the first case series, published by Inoue *et al*[69] in 2014. In ARMS, endoscopic resection of the gastric cardiac mucosa is performed to reduce the opening

of the GEJ. Initial ARMS cases were performed by endoscopic submucosal dissection, but subsequent reports indicated that cap- or band-assisted mucosal resection is faster, easier to perform, and equally effective[70-72]. ARMS has been suggested to suppress the backflow of gastric content and enhance the GEJ flap valve mechanism, but the underlying anti-reflux mechanism is poorly understood[72]. A RCT conducted in animals found that ARMS increased the pressure and volume required to induce fluid passage from the gastric cavity to the esophagus[73]. One clinical study revealed that ARMS increased the integrated relaxation pressure and LES resting pressure but decreased GEJ distensibility, which could hypothetically reduce the frequency of TLESR[72,74].

In 2020, Inoue *et al*[75] and Hernández Mondragón *et al*[76] proposed that ablation of the gastric cardiac mucosa by argon plasma coagulation (forced mode 100 W) or a coagulation current applied by an endoknive (spray coagulation 50 W, effect 2) can also induce scar formation and yield similar clinical outcomes. This approach, named anti-reflux mucosal ablation (ARMA), is intended to simplify the procedure, reduce the risk of perforation, and facilitate the retreatment of patients who have failed ARMS.

In addition to their technical simplicity, ARMS and ARMA do not require costly add-on devices and can be performed in a standard endoscopy room[72,76]. Key points during ARMS and ARMA are adequate submucosal injection to prevent perforation and the sparing of a rim of healthy mucosa to minimize the risk of GEJ stenosis**.** The procedure is not standardized, but most authors spare the esophageal mucosa and perform a gastric cardia 270°-320° treatment or mimic a "butterfly" shape by sparing 1 cm of normal mucosa along the greater and lesser curvature[72,75-77].

In total, 15 nonrandomized studies (12 on ARMS[69-72,74,77-83] and three on ARMA[75,76,84]) comprising 461 patients have evaluated the safety and effectiveness of these techniques (Tables 1 and 3). Follow-up ranged from 2 mo to a maximum of 3 years (in two studies[72,76]). Clinical success ranged from 58% to 100% at 2-6 mo[81, 83] and from 72% to 76% at 3 years[72,76]. Dysphagia was the most common adverse event, occurring in about 5% to 10% of the patients. In contrast to what occurs with dysphagia associated with ARS[85], ARMS- and ARMA-associated dysphagia can be easily treated by small-caliber balloon dilation and does not necessarily compromise clinical success[72,76]. Gastrointestinal perforation is the most feared complication and has been reported in four patients treated with ARMS[72,77,78] and in none treated with ARMA. Given the lack of RCT and long-term data, these techniques should be viewed as experimental and reserved for patients included in research protocols.

Band ligation techniques

Three studies have assessed the outcomes of rubber band placement at the GEJ to reduce the width of the opening of the gastric cardia. Seleem *et al*[86] performed a RCT that included 150 patients with refractory GERD. The number of bands applied and the frequency of endoscopic sessions were determined according to the narrowing of the GEJ during banding. A maximum of four bands per session were allowed. Follow-up at 1 year showed a significant improvement in GERD-HRQL score and the number of reflux episodes. Mild dysphagia (25.3%) and epigastric pain (40%) were the most common adverse events, but no SAEs were recorded[86]. Hu *et al*[87] also reported favorable subjective and 24-h pH-metry outcomes in a case series of 13 patients and named the procedure "peroral endoscopic cardial constriction". The authors placed two single-band ligation devices (Fujinon, Tokyo, Japan) at the greater and lesser curvatures, close to the Z line. The first band was placed approximately 1.0 cm above the cardia along the lesser curvature, whereas the second band was delivered 1.0 cm above the greater curvature[87]. Finally, a clip was placed at the base of the bands to minimize the risk of band slippage. In 2020, another Chinese group reported favorable results with this technique in a nonrandomized study of 60 patients, with the approach now named "clip band ligation anti-reflux therapy (C-BLART)"[88] (Tables 1 and 3).

Because the above-mentioned RCT does not adhere to the Consolidated Standards of Reporting Trials quality requirements and the two case series were noncontrolled and included a limited number of patients, the technique should currently be viewed as experimental.

FUTURE DIRECTIONS

The history of endoscopic therapies for GERD is replete with encouraging preclinical studies and case series that fail to clear the hurdle of long-term and well-designed

RCTs. The main underlying reasons are the complex and multifactorial pathophysiology of GERD and the often short-lived anatomical changes induced by endoscopic therapies. Moreover, many endoscopic techniques require expensive add-on devices and cumbersome technical steps that have limited their popularization. To complicate further this issue, patient selection has been heterogeneous, and we lack consensus regarding the definition of clinical success or the admissible thresholds of cost and adverse events. Future endoscopic therapies and GERD research should bear all of this in mind.

The first consideration is that only a subset of well-selected GERD patients are good candidates for endoscopic therapies because current techniques remain unable to fix the hiatus, enhance esophageal motility, or normalize LES competence. Artificial intelligence through knowledge-based clinical decision support systems could be of help in the future for improving patient selection. Combined approaches that consider more than one GERD mechanism have been proposed to address this issue, such as a combination of ARMS with a plication method[89] or of TIF with laparoscopic HH repair[90]. Second, technical feasibility is critical for introducing a procedure into clinical practice. The learning curve of anti-reflux endoscopic therapies has not been well-described, and scientific societies have not published curricula documents to guide training. Band ligation, ARMS, and, more recently, ARMA are at very early stages but represent an attractive option from this perspective. Our group is currently performing a double-blind RCT to assess the clinical success and safety of ARMA[91]. Third, patient-reported outcomes are increasingly being recognized by clinicians, regulatory agencies, and patients as highly valuable tools to assess the impact of new interventions. Thus, we believe that studies should place symptoms and GERD-related quality of life as primary endpoints. A "black or white" perspective for clinical success does not reflect the complexity of GERD patients, and partial but significant improvements should also be taken into account. This makes anti-reflux endoscopy not only an alternative to PPIs, but also a complementary tool that can reduce their consumption and partially improve quality of life. A > 50% drop in the GERD-HRQL score or in other validated clinical questionnaires has been used in recent RCTs and appears to be a reasonable approach[52,53]. In addition, more objective GERD parameters (24-h pH-impedance testing, endoscopic esophagitis) and sham/placebo arms are needed to support subjective improvements. Outcome definitions should be in line with recent international consensus[26,27,92,93]. RCTs should include long-term follow-up as part of the trial or as a post-RCT prospective observational phase to assess durability. Finally, endoscopic therapies seem cost-effective, but we need more comparative data with PPI and surgery[94].

CONCLUSION

Endoscopic therapy for GERD aims to offer an alternative to PPIs and ARS in patients without significant diaphragmatic crura impairment. TIF, the technique with the largest body of evidence, has been proven to improve GERD symptoms and acid exposure time and reduce PPI consumption. Nonablative radiofrequency (Stretta®) is the method with the lowest rate of SAEs, but its efficacy has been called into question in recent meta-analyses. Band ligation techniques, ARMS, ARMA, and new plication devices have shown promising results in initial reports and RCTs are eagerly awaited. Careful patient selection, ongoing technical refinements, and RCTs with long-term data are the roadmap to unveil the potential of minimally invasive anti-reflux endoscopic techniques.

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ORIGINAL ARTICLE

Basic Study

Cold exposure and capsaicin promote 1,2-dimethylhyrazine-induced colon carcinogenesis in rats correlates with extracellular matrix remodeling

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Abstract

BACKGROUND

Extracellular matrix (ECM) remodeling and stiffening, which are correlated with tumor malignancy, drives tumor development. However, the relationship between ECM remodeling and rat experimental model of 1,2-dimethylhyrazine (DMH)-induced colorectal cancer (CRC) imposed by cold and capsaicin exposure remains unclear.

AIM

To explore the effects of cold exposure and capsaicin on ECM remodeling and ECM enzymes in DMH-induced CRC.

METHODS

For histopathological analysis, the sections of colon tissues were stained with hematoxylin and eosin, Masson's trichrome, Picrosirius red, and Weigert's Resorcin-Fuchsin to observe the remodeling of collagen and elastin. Additionally, the protein expression level of type I collagen (COL I), type 3 collagen (COL III0, elastin, matrix metalloproteinase (MMP) 1, MMP2, MMP9, and tissue-specific

nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Cold exposure and Capsaicin promote 1,2-dimethylhydrazine-induced colon carcinogenesis in rats correlates with extracellular matrix remodeling".

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matrix metalloproteinase 1 (TIMP1) was assessed by immunohistochemistry. The messenger RNA (mRNA) levels of COL I, COL III, elastin, and lysyl oxidase-like-2 (LOXL2) in the colon tissues of rats was measured by reverse-transcriptase quantitative polymerase chain reaction.

RESULTS

Although no differences were observed in the proportion of adenomas, a trend towards the increase of invasive tumors was observed in the cold and capsaicin group. The cold exposure group had a metastasis rate compared with the other groups. Additionally, abnormal accumulation of both collagen and elastin was observed in the cold exposure and capsaicin group. Specifically, collagen quantitative analysis showed increased length, width, angle, and straightness compared with the DMH group. Collagen deposition and straightness were significantly increased in the cold exposure group compared with the capsaicin group. Cold exposure and capsaicin significantly increased the protein levels of COL I, elastin, and LOXL2 along with increases in their mRNA levels in the colon tissues compared with the DMH group, while COL III did not show a significant difference. Furthermore, in immunohistochemical evaluations, MMP1, MMP2, MMP9, and TIMP1 staining increased in the cold exposure and capsaicin group compared with the DMH group.

CONCLUSION

These results suggest that chronic cold and capsaicin exposure further increased the deposition of collagen and elastin in the colonic tissue. Increased COL I and elastin mRNA and protein levels expression may account for the enhanced ECM remodel and stiffness variations of colon tissue. The upregulated expression of the LOXL2 and physiological imbalance between MMP/TIMP activation and deactivation could contribute to the progression of the CRC resulting from cold and capsaicin exposure.

Key Words: Colon cancer; Cold exposure; Capsaicin; Extracellular matrix remodeling; Extracellular matrix enzymes

Core Tip: In this study, we discovered that remodeling of extracellular matrix (ECM) plays an important role in the progression of colorectal cancer (CRC). These results suggest that increased stiffness of colonic tissue and the remodeling of ECM mediated by ECM enzymes resulting from cold and capsaicin exposure predisposes an environment suitable for CRC development and progression. To target ECM in CRC tumor tissue could represent a novel potential therapeutic strategy.

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INTRODUCTION

Colorectal cancer (CRC), a common cause of cancer deaths in the world, is a multifactorial disease driven by genetic predisposition, epigenetic alterations, and environmental factors[1]. Only a minority of CRC is caused by the accumulation of genetic epigenetic alterations, while the majority is linked to environmental factors such as dietary intake, alcohol consumption, and ambient environment[2,3]. Increasing epidemiological data have indicated that cold weather might be associated with an increased occurrence of cancer[4]. Additionally, the consumption of chili-pepper and cancer incidence have a positive correlation[5,6]. However, the mechanisms under-

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lying the effects of cold exposure and capsaicin in 1,2-dimethylhyrazine (DMH)-induced CRC tumorigenesis and progression remain poorly understood. Extracellular matrix (ECM) is a non-cellular structure that is essential for the maintenance of normal tissue and organ function and disease pathophysiology[7]. Collagen is a major component of the ECM. It provides cellular components with physical support and is an important contributor to tumor growth and progression[8]. Tumor progression is accompanied by the dysregulation of collagen structure and deposition. Tumor associated-collagen is usually compacted to thick collagen bundles and the anisotropic arrangement of relatively straight in the matrix of malignancies compared with healthy tissues[9,10]. In clinical samples of breast tumors, collagen deposition increased, and linearization and thickening of collagen occurred; these processes can be linked to poor prognosis and high risk of mortality[11,12].

Elastin is another important fibrous ECM protein that provides elastic recoil to tissue. Importantly, excessive accumulation of ECM, particularly collagen and elastin, gradually leads to progressive organ fibrosis[13]. The fibrosis results in tissue stiffness and can predispose tissue to malignancy. Several human studies indicated that patients with liver fibrosis and stiffness are positively correlated with the risk hepatocellular carcinoma[14]. ECM remodeling is mainly orchestrated by ECM modifying enzymes such as lysyl oxidase-like-2 (LOXL2), matrix metalloproteinases (MMP), and tissue-specific matrix metalloproteinase inhibitors (TIMPs)[15]. LOXL2 is a key factor in ECM remodeling and is a copper-dependent amine oxidase that catalyzes the cross-linking of collagen or elastin in the ECM and thus regulates the tensile strength of tissues. LOXL2 causes disorganization and composition of ECM, resulting in many pathological conditions, including fibrosis and cancer[16]. Active LOXL2 is involved in stiffness-associated cancer progression, whereas inhibition of LOXL2 result in less collagen cross-linking and impeded cancer progression[17]. Moreover, LOXL2 expression is overexpressed in many types of tumors and is associated with poor prognosis[18-20].

MMP has been implicated in cancer development, progression, invasiveness, and dissemination by promoting a protumorigenic microenvironment and modulating the ECM and intercellular junctions[21]. MMPs and TIMPs are the main enzymes involved in the regulation of ECM remodeling and collagen degradation process[22]. Their expression and activity are upregulated in almost all human cancers with disparate changes, and this phenomenon is associated with advanced tumor stage, poor prognosis, and decreased overall survival rate[23,24]. Increased MMP expression/activity or decreased TIMPs could lead to MMP/TIMP imbalance, resulting in various pathological conditions including fibrosis and cancers[25].

Limited information, however, is available about ECM remolding and ECM enzyme activity in the progression of experimental colorectal malignancy. We have previously shown that cold exposure and long-term administration of capsaicin at a low dose further promote the development and progression of CRC[26]. However, the specific mechanisms underlying cold and capsaicin exposure tumor promotion remained unknown. This study aimed to investigate the effects of cold exposure and capsaicin on ECM remodeling and ECM enzymes in DMH-induced CRC. Moreover, we determined whether excessive ECM deposition, particular whether collagen and elastin and dysregulation of ECM enzymes expression and/or secretion in rat treatment with cold exposure, could further stiffen the colon tissues and disrupt the intestinal morphogenesis to exacerbate the experimental colorectal malignancy.

MATERIALS AND METHODS

Experimental design in adult male rats

Wistar rats weighing 200-250 g (6-wk-old) were obtained from Experimental Animal Center in Guangzhou University of Chinese Medicine. Animals were housed in plastic cages under a controlled environment (24 ± 2 °C, 50% ± 5% humidity, 12 h/12 h light-dark cycle) with *ad libitum* food and water access. All the experimental protocols were approved by the Institutional Animal Ethics Committee of the Guangzhou University of Chinese Medicine (No. 20130001). Briefly, after 3 d of acclimation, the animals were randomly assigned into four groups (n = 10). Rats in group A received no treatment and served as control. Five weeks later, rats in groups B-D received subcutaneous injection of DMH (25 mg/kg) once a week for 12 wk. In addition to DMH, Group C rats received cold distilled water (10 mg/kg) until the end of 38 wk. Group D rats were given capsaicin (0.9 mg/mL) every day throughout the experiment. By the end of the week, 10 rats from each group were sacrificed. For macroscopic evaluation of the

incidence of polyps at the end of the experimental period, rats were sacrificed and colons were incised and washed with physiological saline. Then cleaned colons were cut opened longitudinally and the total number of polyps/tumors was carefully counted and later verified with histopathological examination. The counting and histopathological analysis of gross macroscopic neoplastic lesions was carried out by two investigators from this study. If the histopathological types of these two investigators were different, then tumor histology was classified as adenomas and adenocarcinomas by one pathologist under blinded conditions from the Pathology Department in Guangzhou University of Chinese Medicine. Microscope findings were classified as adenomas and adenocarcinomas according to previous criteria described by Jikihara *et al*[27]. Tumor incidence is the percentage of rat bearing the indicated type of tumor.

Histopathological staining

All specimens were fixed in 4% paraformaldehyde solution for 24 h and embedded in paraffin and processed by standard histological processing techniques. Serial issue sections (8-μm thick) were obtained from each sample with the microtome and then were stained with hematoxylin and eosin (HE), picrosirius red, Masson's trichrome (MT), and Weigert's Resorcin-Fuschin (WRF). For Picrosirius red staining, sections were stained in picrosirius red solution 0.1) (Sirius red F3B; Sigma-Aldrich Co., St Louis, MO, United States) in a saturated aqueous solution of picric acid for 1 h at room temperature for collagen bundle staining. Images were subsequently analyzed using ImageJ to calculate the fiber density, which was measured as image % area coverage. MT was performed according to the manufacture's protocol including Weigert's Iron Hematoxylin Solution, Ponceau acid fuchsin, and Aniline Blue as reagents. The collagen volume fraction was measured by ImageJ software and calculated as the proportion of blue positive areas in the total section areas. The process of WRF used reagents and kits from Solarbio (Beijing, China). Sections were then mounted for observation under polarized light microscopy (NIKON Eclipse Ci, Tokyo, Japan) and light microscopy, respectively. Three microphotographs of the reticular dermis were taken with a 400 × magnification with light microscopy and polarized microscopy, respectively. Digitized images of histological sections obtained under final magnification of × 400 were analyzed using the Image-Pro Plus 4.5 software.

Collagen fiber analysis

CT-FIRE, an open-resource software (http://Loci.wisc.edu/software/cifire), was used as previously described to quantify automatically collagen fibers[28]. The quantitative parameters included alignment of collagen fibers as well as individual length, straightness, and width. These features of collagen fibers are widely used to investigate collagen organization in a various of cancer[29]. All the picrosirius red images were converted to 8-bit images and threshold values between 10-255 to eliminate background noise using FIJI ImageJ[30]. These images were uploaded to CT-FIRE; collagen fiber extraction parameters were set to default parameters.

Real-time quantitative reverse-transcriptase polymerase chain reaction

Total RNA extracts of colon tissues were prepared using TRIZOL reagent (TaKaRa, Kusatsu, Japan). RNA (1 μg) was reverse transcribed in a 20 μL reaction mixture using Prime Script RT Master Mix (TaKaRa). The purity of total RNA was evaluated by measuring the concentration and $OD_{260:280}$ values with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The mRNA levels of collagen type 1, alpha1 (COL1A1), collagen type 3, alpha1 (COL3A1), LOXL2, and elastin in colon mucosa were assessed using a Step One Plus real-time polymerase chain reaction system (CFX384TM Real-time System; Thermo Fisher Scientific). The relative levels of gene expression were enumerated using the comparative formula 2-ΔΔCt. The primer sequences used in this polymerase chain reaction amplification were as follows: 5′-AGCCATGTACGTAGCCATCC-3′/3′-ACCCTCATAGATGGGCACAG-5′ for β-actin; 5′-AGGCATAAAGGGTCATCGTGGCTT-3′/3′-AGTCCATCTTTGC-CAGGAGAACCA-5′ for COL1a1; 5′-GGTTTGGAGAATCTATGAATGGTGG-3′/3′-GCTGGAAAGAAGTCTGAGGAAGG-5′ for Col3a1; 5′-AGCCTATAAGCCG-GAGCAAC-3′/3′-GTCCCACTTGTCATCGCAGA-5 'for LOXL2; 5′-CGCCTGTAAT-GCCTCCAATC-3′/3′-AGCAGCTAAAGCAGCGAAGT-5′ for elastin.

Immunohistochemistry

Colonic tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol/water. The sections were placed in a 95 °C antigen retrieval solution (citrate buffer; PH 6.0) for 15 min. After cooling in retrieval solutions for 20 min at

Table 1 Incidence of various tumors induced in different treatment groups

Group	Total number of tumors	Adenoma incidence, %			Adenocarcinoma incidence, %				With lymphytic metastasis (%)
		Mild	Moderate	Severe	Well-differentiated	Moderate-differentiated	Poor-differentiated	Mucinous	Metastasis rate
Control	-	-	-	-	-	-	-	-	-
DMH	23	2/23 (8.7)	2/23 (8.7)	3/23 (13)	12/23 (52.2)	4/23 (17.4)	3/23 (13.0)	-	-
Cold exposure	38	1/38 (2.6)	2/38 (5.3)	3/38 (7.8)	5/38 (15.8)[b]	8/38 (21.1)	15/38 (36.8)[a]	4/38 (10.5)	20.0
Capsaicin	34	2/31 (5.9)	1/34 (2.9)	4/31 (12.9)	7/31(22.6)[a]	12/31 (38.7)	7/31(22.6)	1/31 (3.2)	-

Values are expressed as the proportion of lesions-bearing rats. n = 10 rats/group. Incidence data was analyzed by using chi-square or Fisher's exact test.
[a]$P < 0.05$.
[b]$P < 0.01$.
1,2-dimethylhyrazine compared with cold exposure and capsaicin-treated group. DMH: 1,2-Dimethylhyrazine.

room temperature, the slides were treated with hydrogen peroxide for 10 min to block endogenous peroxidase activity. Primary rabbit anti-histone polyclonal antibodies were applied for 14 h at 4 °C overnight at the following dilutions: Type I collagen (COL I) (1:500; ab34710; Abcam, Cambridge, United Kingdom), type III collagen (COL III) (1:200, ab7778; Abcam), LOXL2 (1:400), elastin (1:600; ab217356; Abcam), MMP1 (1:500; 10371-2-AP; Proteintech, Rosemont, IL, United States), MMP2 (1:200; ab86607; Abcam), MMP9 (1:800; ab38898; Abcam), and TIMP1 (1:600, ab61224; Abcam). The next day, biotin-conjugated secondary antibody and streptavidin-biotin peroxidase were applied each for 20 min. 3,3'-Diaminobenzidine tetrahydrochloride (0.05%) was used as the substrate, and nuclear contrast was performed using hematoxylin counter-staining. Each section was analyzed in three different fields using Image Pro Plus software. The density of yellow reflects the expression levels of target proteins. Integral optical density sum / area SUM was applied to quantify the relative expression of COL I, COL III, LOXL2, elastin, MMP1, MMP2, MMP9, and TIMP1.

Statistical analysis

All the data are summarized as mean ± SD, and data were analyzed using SPSS 23.0 statistical software. We performed the data with a one-way analysis of variance with the post-hoc comparison by the L.S.D. method and Fisher's exact test was employed to compare tumor incidence. Differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

Macroscopic and pathological observation study of colon tumor

No visible colon tumor was found in the normal control. We observed findings such as colonic mucosal thickening, stiffness, and not tiled completely on filter paper in the majority of rats of the cold exposure group (Figure 1C). As shown in Figure 1B, the length of colon in the DMH and normal group was not significantly different. However, the length of the colon in the cold exposure and capsaicin group was shorter than that of DMH group. The pathological classification of colonic tumors in each group is shown in Table 1. No difference was observed in the proportion of adenomas among groups. In the DMH-induced group of animals, most tumors had well-differentiated tubular adenocarcinomas. Invasive tumors increased in the cold and capsaicin group. Histopathological analysis showed in the cold exposure group that an evident malignant transformation occurred in the colon with the features of poor-differentiated mucinous adenocarcinomas, and some of the glands were filled with mucinous material (Figure 2C). In addition, the mesenteric lymph nodes of rats in each group were stained with hematoxylin and eosin, and the lymphatic metastasis was observed under light microscope (Figure 2B). No lymphatic metastasis was observed in the DMH and capsaicin group, while a mesocolic lymph node was totally replaced by

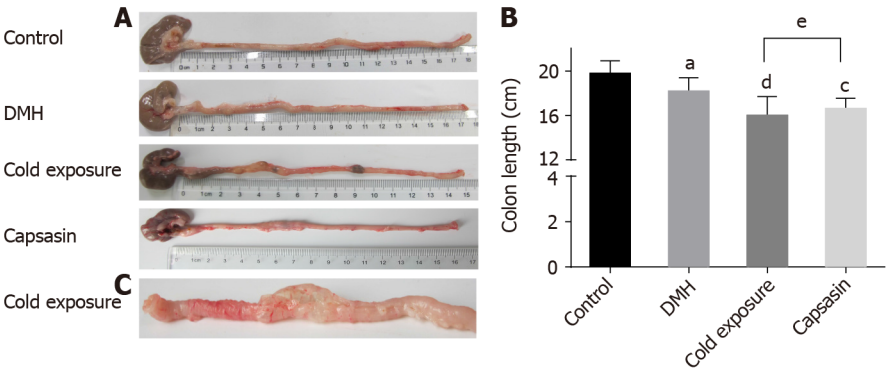

Figure 1 Colonic morphology of rats in different groups. A and B: Changes in colon length and colonic morphology; C: Stiff colonic tissues in cold exposure group. [a]*P* < 0.05, control compared with 1,2-dimethylhyrazine (DMH); [c]*P* < 0.05, [d]*P* < 0.01, DMH compared with cold exposure and capsaicin-treated group; [e]*P* < 0.05, cold exposure compared with capsaicin-treated group.

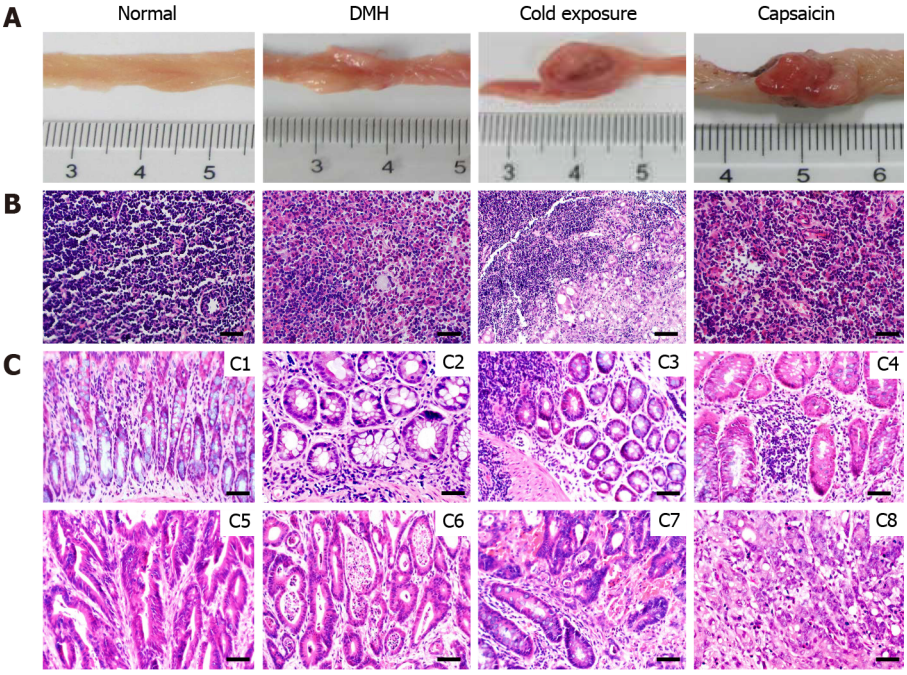

Figure 2 Pathological observation and lymph node metastases of different groups. A: Macroscopic image of the colonic tumors; B: Representative sections stained with hematoxylin and eosin (HE) showing the histopathology of the mesocolic lymph node in the different groups; C: Representative sections stained with HE showing the histopathology of the colonic mucosa in the different groups. Normal architecture of colon was observed in the control groups (C1), adenoma with mild dysplasia with massive infiltration of inflammatory cells (C2). Histology of adenoma with moderate dysplasia in cold exposure groups (C3). Histology of adenoma with severe dysplasia (C4). Histology of well-differentiated tubular adenocarcinomas (C5). Histology of Moderately differentiated adenocarcinomas (C6). Histology of Poorly differentiated adenocarcinomas (C7). Histology of Mucinous adenocarcinoma with signet ring cells (C8) (HE staining, × 400, scalar bar 20 μm). DMH: 1,2-Dimethylhyrazine.

metastatic cancer tissue in the cold exposure group; the lymph node metastasis rate was 20.0% (Table 1, Figures 1 and 2).

Alterations in collagens after cold exposure and capsaicin

Colon tissue sections were stained by MT and picrosirius red to identify the total collagen in the colon mucosa. As shown in Figure 3A, there were few collagen fibers in normal colonic mucosa. After DMH treatment, wave shape collagens stained blue were markedly increased around the glands, and this was increased further in the cold exposure and capsaicin treatment group. The collagen density was quantified using ImageJ, and it was significantly increased in the colonic tissue of cold exposure and capsaicin group. This excessive collagen deposition was further confirmed by picrosirius red staining. As shown in Figure 3B, picrosirius red staining revealed in normal tissue the collagen fibers with sparse deposition composed of thin collagen fibers. The collagen fibers in the DMH group were denser than that in normal collagen

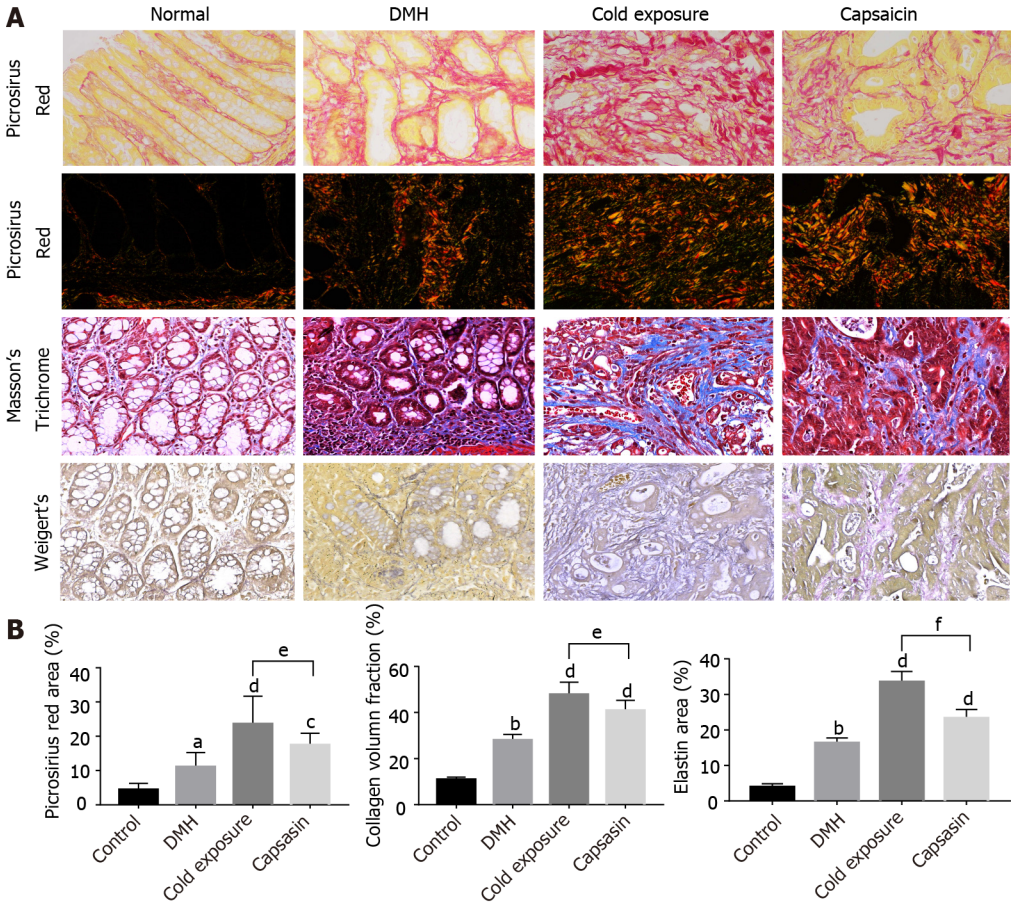

Figure 3 Changes in extracellular matrix components (collagen fibers and elastin) in colonic mucosa of different treatment groups. A: Representative photographs of colonic tissues in rats of normal, 1,2-dimethylhyrazine (DMH), cold exposure and capsaicin groups using Masson's trichrome: collagen (blue), nuclei and cytoplasm (red); picrosirius red in bright-field: collagen (red); polarized light: collagen (yellow-orange to green birefringence) and Weigert's Resorcin-Fuschin: elastin (blue-black), myofibers (yellow). Magnification, × 400, scalar bar 20 μm; B: Quantitative analysis of picrosirius red staining, trichrome and Weigert's staining as a measure of collagen and elastin density.

fibers. In the capsaicin treatment group, collagen fibers showed an evident increase and were crosslinked into bundles. On the other hand, the cold exposure group apparently displayed an increased amount of collagen fibers with heterogeneous thickness and alignment. The collagen in the cold exposure and capsaicin group exhibited a predominant reddish or yellow-orange. The structure and organization of collagen fibers were evaluated in colon tissue sections by quantifying the polarization microscopy images. As shown in Figure 4A, visualized collagen fibers were extracted and analyzed for fiber width, angle, length, and straightness using CT-FIRE software. As shown in Figure 4B, compared with the DMH group, collagen fibers in the cold exposure and capsaicin group showed a significant increase in angle, length, width, and straightness. These results revealed that cold exposure and capsaicin induced a progressive increase in the content and orientation of collagen fibers in CRC as a function of malignancy.

Alterations in elastin after cold exposure and capsaicin

Treatment WRF was used to identify the elastin fibers, which were stained black. As shown in Figure 3A, elastin was hardly expressed in the colonic mucosa of the normal rats. After treatment with DMH, the elastin fibers aligned surrounding the epithelium and stroma. After cold exposure and capsaicin treatment, the amount of elastin fibers increased, and thick elastic fibers was found highly disorganized between the gland compared with their respective control and DMH groups. Alterations in the mRNA levels of COL I, COL III, LOXL2, and elastin. The expression levels of COL I, COL III, LOXL2, and elastin mRNAs in colonic tissue are shown in Figure 5. The COL I, LOXL2, and elastin mRNA levels were higher in the DMH-induced cancer group than in the control group. In comparison with the DMH group, a significant increase was detected both in the cold exposure and capsaicin treatment group, but the mRNA levels of COL III were not significantly different between DMH and capsaicin ex-

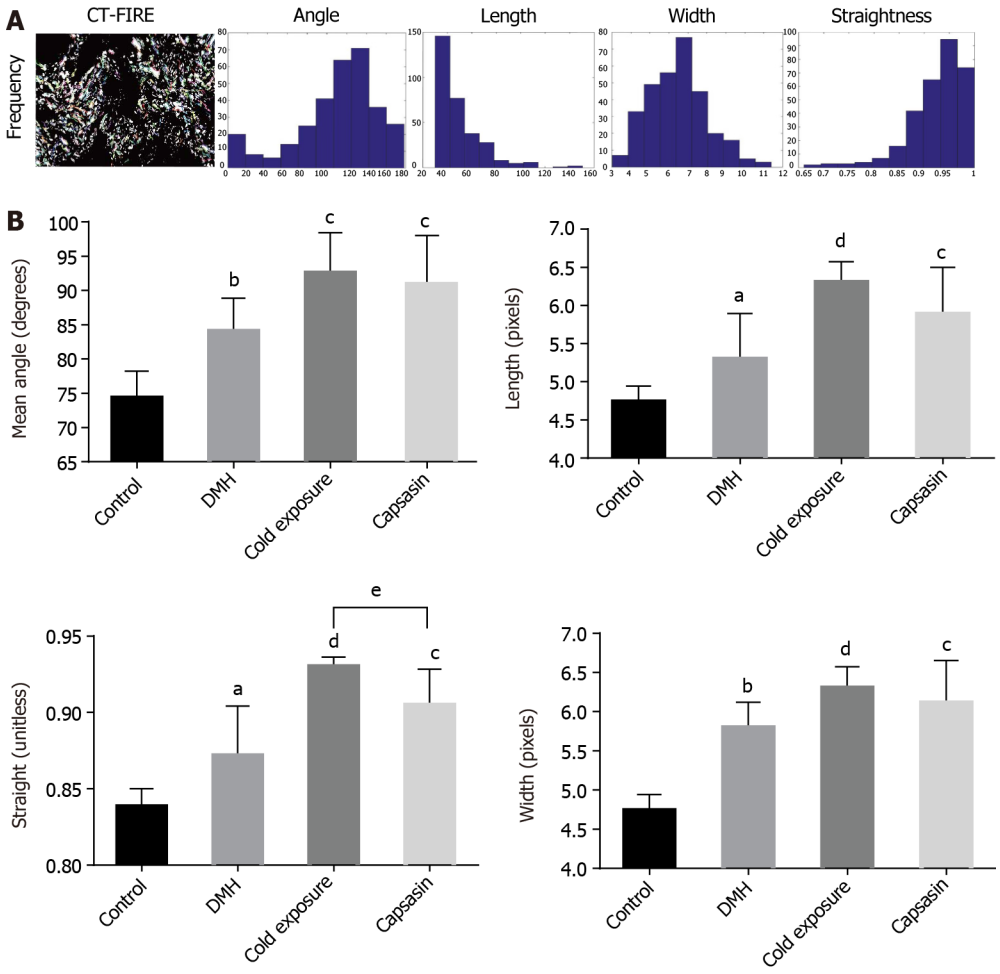

Figure 4 Collagen fibers were automatically extracted for analysis using open-source software CT-FIRE. A: Histograms were generated to show the distribution of various parameters in each polarized light microscopy imaging; B: Quantitative analysis of collagen fibers from polarized light microscopy imaging in the colonic mucosa of different treatment groups. Data are mean ± SE of three images per tissues region. $^{a}P < 0.05$, $^{b}P < 0.01$, control compared with 1,2-dimethylhyrazine (DMH); $^{c}P < 0.05$, $^{d}P < 0.01$, DMH compared with cold exposure and capsaicin-treated group; $^{e}P < 0.05$, Cold exposure compared with capsaicin-treated group.

posure group.

Alterations in the protein levels of COL I, COL III, LOXL2, and elastin

The expression levels of COL I, COL III, LOXL2, and elastin in colonic tissue are shown in Figure 6A and B. The protein expression levels of collagen type I, III, LOXL2, and elastin were significantly elevated in colonic tissue from DMH-treated rats in comparison to the control group. The expression level of proteins in the cold exposure and capsaicin treatment group increased. COL I and LOXL2 levels were significantly higher in the cold exposure group, but no statistical difference was observed in the change of COL III and elastin between cold exposure and capsaicin group.

Alterations in the protein levels of MMP1, MMP2, MMP9, and TIMP1

The expression levels of MMP1, MMP2, MMP9, and TIMP1 in colonic tissue are shown in Figure 7. Significantly elevated MMP1, MMP2, MMP9, and TIMP1 immunoreactivity was observed in DMH-treated rats compared with the control group. The expression levels of proteins in the cold and capsaicin group increased compared with the DMH group. In comparison with the capsaicin group, the expression of MMP2, MMP9, and TIMP1 increased in the cold exposure group.

DISCUSSION

ECM has been increasingly considered as an important regulator at diverse aspects of tumor initiation, promotion, neoplastic transformation, invasion, and metastasis[31].

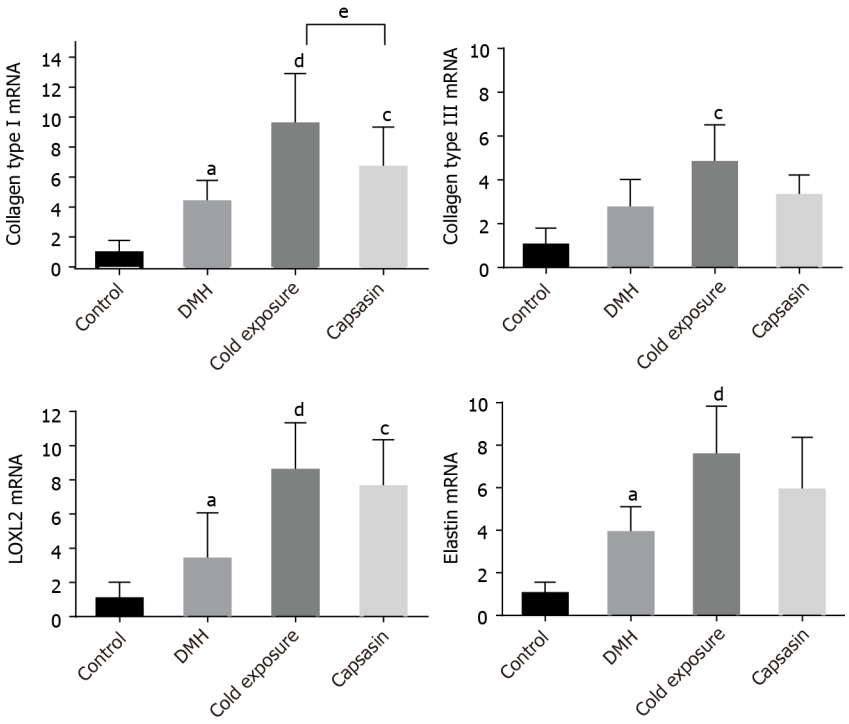

Figure 5 The mRNA expression levels of collagen type I, III, elastin and lysyl oxidase-like-2 in the colon tissues of rats in different groups. Data were presented as the mean ± SD form six independent experiments. [a]*P* < 0.05, control compared with 1,2-dimethylhyrazine (DMH); [c]*P* < 0.05, [d]*P* < 0.01, DMH compared with cold exposure and capsaicin-treated group; [e]*P* < 0.05, cold exposure compared with capsaicin-treated group. LOXL2: Lysyl oxidase-like-2.

Furthermore, ECM remodeling is a consequence of or increases risk for malignant transformation of colonic, hepatic, pulmonary, and pancreatic cells[32,33]. Collagen and elastin are the major components of ECM, and their excessive deposition has been implicated in a number of diseases, particularly fibrosis and cancer. However, the morphology and structure of collagen and elastin fibers in the animal models of CRC remains unclear. In the present study, we analyzed the morphology and structure of collagen and elastin fibers in rat experimental model of DMH-induced CRC imposed by cold and capsaicin exposure. Results showed an association between collagen expression or ECM modifying enzymes and CRC development, thus supporting ECM remodeling is highly relevant to CRC cancer progression. Tumor tissue often exhibits fibrosis, and this fibrotic state is characterized by the excessive deposition of collagen and elastin[34].

Fibrosis can develop in nearly any organ, and it is an important driver of tissue stiffness and increases the risk of malignancy[35]. In fibrotic kidney biopsy specimens or multiple experimental kidney fibrosis rodent models, the accumulation of elastin can be observed in renal tissue[36]. In human fibrosis of the liver, kidney, and pancreas, the ECM on average becomes stiffer than normal. Our previous study indicated in human CRC that the collagen development features numerous changes in composition and organization compared with normal colonic tissues[37]. In the present study, we found that collagen components were quantitatively and qualitatively changed in the rat experimental model of CRC. By using picrosirius red, MT, and WRF staining, we revealed a marked increase in collagen and elastin deposition in rats exposed to cold and capsaicin treatment. Furthermore, they were more orderly organized based on the collagen fibers being more aligned with each other, longer, wider, and slightly straighter.

The structure, orientation, and physical properties of collagen regulate the aggressive behavior of cancer. For example, in glioblastoma, Pointer *et al*[38] showed that patients with more organized glioblastoma multiforme collagen survive longer than patients with less organized glioblastoma multiforme collagen. Zhou *et al*[39] also demonstrated that the increased density, length, or width of collagen negatively affects patients with gastric cancer prognosis. The stromal tissue in CRC has also shown that an increase in the collagen content of the ECM increases mechanical stiffness, which predisposes to aggressive CRC[40]. In human breast cancer, linear organization and relatively straight collagen that facilitates migration of tumor cells indicate poor cancer outcome[41]. Similarly, our data indicated that cold and capsaicin exposure increased

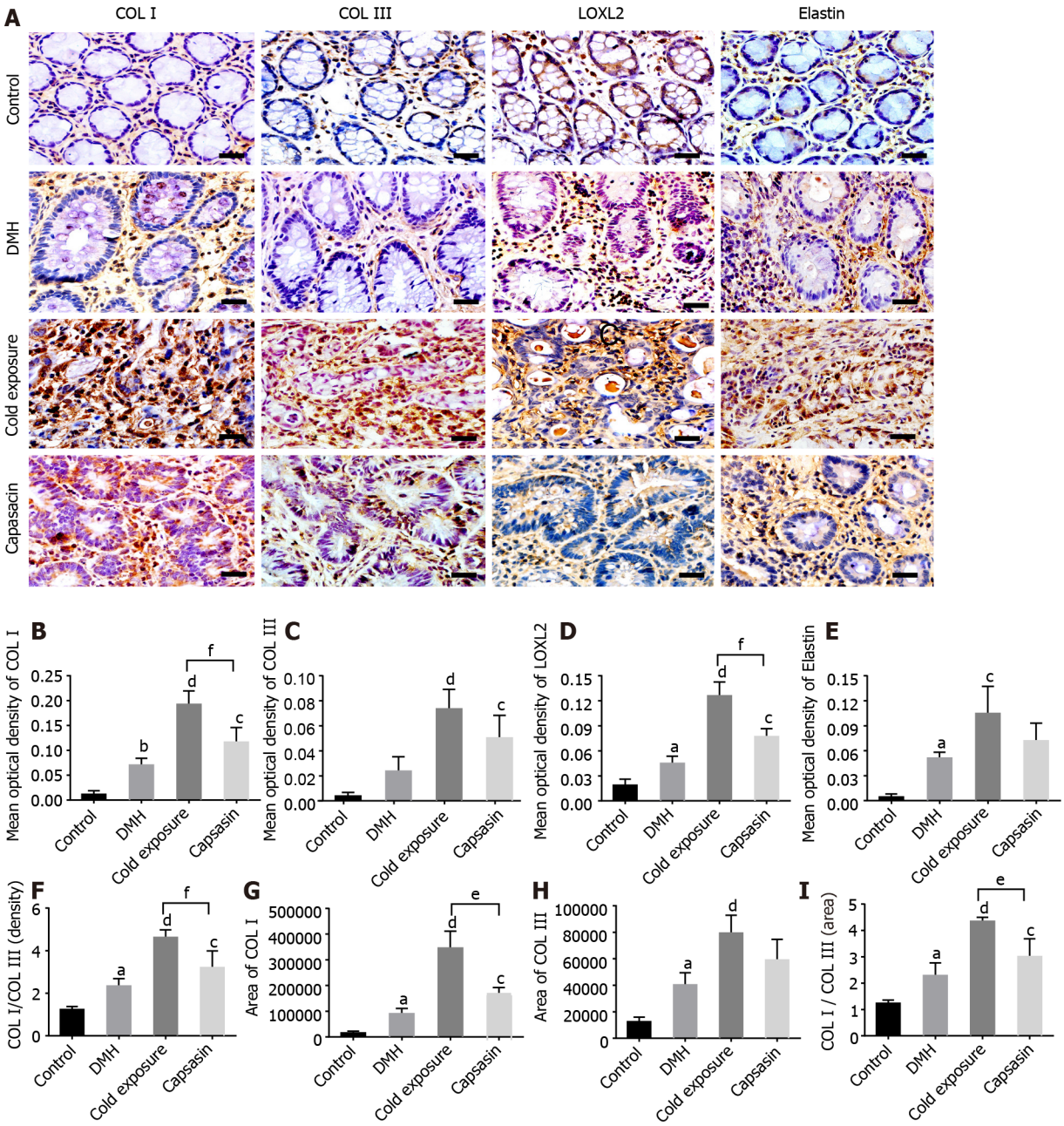

Figure 6 Changes in collagen, elastin and lysyl oxidase-like-2 proteins in the colonic tissues of different treatment groups. A: Protein expressions of type I collagen (COL I), type III collagen (COL III), lysyl oxidase-like-2 (LOXL2), and elastin in the colonic tissues *via* immunohistochemical staining. Magnification, × 400, scalar bar 20 μm; B-I: Densitometric analysis of COL I (B), COL III (C), LOXL2 (D), elastin(E), COL I/COL III (F), COL I area (G), COL III area (H), and COL I area/COL III area (I) during immunohistochemical staining. $^{a}P < 0.05$, $^{b}P < 0.01$, control compared with 1,2-dimethylhyrazine (DMH); $^{c}P < 0.05$, $^{d}P < 0.01$, DMH compared with cold exposure and capsaicin-treated group; $^{e}P < 0.05$, $^{f}P < 0.01$, cold exposure compared with capsaicin-treated group.

collagen and elastin deposition, thus triggering alterations in the ECM architecture and organization in the DMH-induced CRC for further tumor development and progression. Furthermore, all parameters of collagen fibers (*e.g.*, density, angle, length, width, and straightness) significantly increased in the cold exposure group and could accurately explain the cold-induced CRC more seriously.

Recently, the relationship between ECM remodeling and malignant transformation of cancer has attracted much attention[42]. COL I is the most abundant protein present in the body. COL I, a major component of collagen, was significantly up-regulated in CRC tissues and showed enhanced CRC migratory capabilities through the overexpression of WNT/planar cell polarity signaling pathway[43]. Moreover, the elevated expression of type I collagen in CRC tissues is correlated to patients with high metastasis that was due to activation of phosphatidylinositol-3-kinase/AKT signaling [44]. Bode *et al*[45] also showed that the expression of COL was increased in malignant colon tissue compared with COL III. Moreover, the elevated expression of COL I has

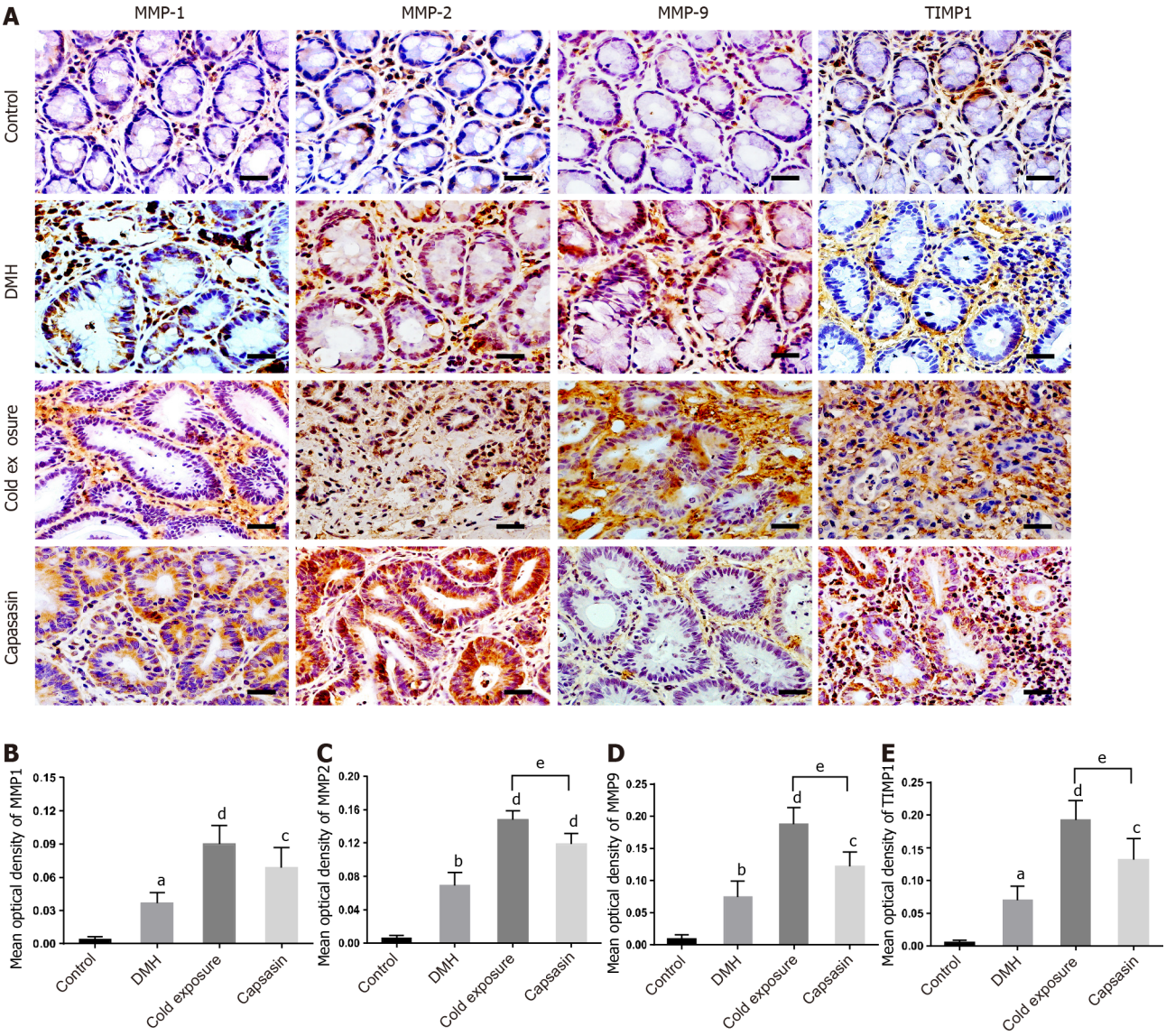

Figure 7 Changes in matrix metalloproteinase 1, matrix metalloproteinase 2, matrix metalloproteinase 9, and tissue-specific matrix metalloproteinase 1 proteins in the colonic tissues of different treatment groups. A: Protein expressions of matrix metalloproteinase (MMP) 1, MMP2, MMP9 and tissue-specific matrix metalloproteinase 1 (TIMP1) in the colonic tissues *via* immunohistochemical staining. Magnification, × 400, scalar bar 20 μm; B-E: Densitometric analysis of MMP1 (B), MMP2 (C), MMP9 (D) and TIMP1 (E) during immunohistochemical staining. $^{a}P < 0.05$, $^{b}P < 0.01$, control compared with 1,2-dimethylhyrazine (DMH); $^{c}P < 0.05$, $^{d}P < 0.01$, DMH compared with cold exposure and capsaicin-treated group; $^{e}P < 0.05$, cold exposure compared with capsaicin-treated group.

been linked to the invasive and aggressive behavior of CRC[46]. In the present study, we also evaluated the expression of COL I in cold exposure and capsaicin treatment CRC colonic tissue. The increase in expression levels of COL I in our study is consistent with other reports in CRC. However, the mRNA level of COL III was not significantly different between the DMH and capsaicin group. In addition, compared with other groups, the COL I/ COL III in cold exposure group was significantly increased. With the increase in collagen expression, distribution area, and collagen ratio, the degree of fibrosis in ECM pathological characteristics was aggravated[47,48]. Therefore, the colonic tissue stiffness was significantly higher than that of the other groups, and the degree of ECM fibrosis in CRC with cold exposure was more serious than that in other groups. ECM remodeling is regulated by ECM enzymes such as LOXL2, MMPs, and TIMPs. ECM-crosslinking enzyme LOXL2 has been implicated in stiffness-associated tumor progression[49]. The LOXL2-mediated collagen cross-linking, both *in vitro* and *in vivo* models of CRC, results in increased tissue stiffness and activation of the focal adhesion kinase/SRC signaling[50].

MMP1, MMP-2, and MMP-9 play a fundamental role in many pathophysiological processes such as cell migration, angiogenesis, and the invasion and metastasis of malignant tumors[51]. TIMPs are the most important physiological inhibitors of MMP,

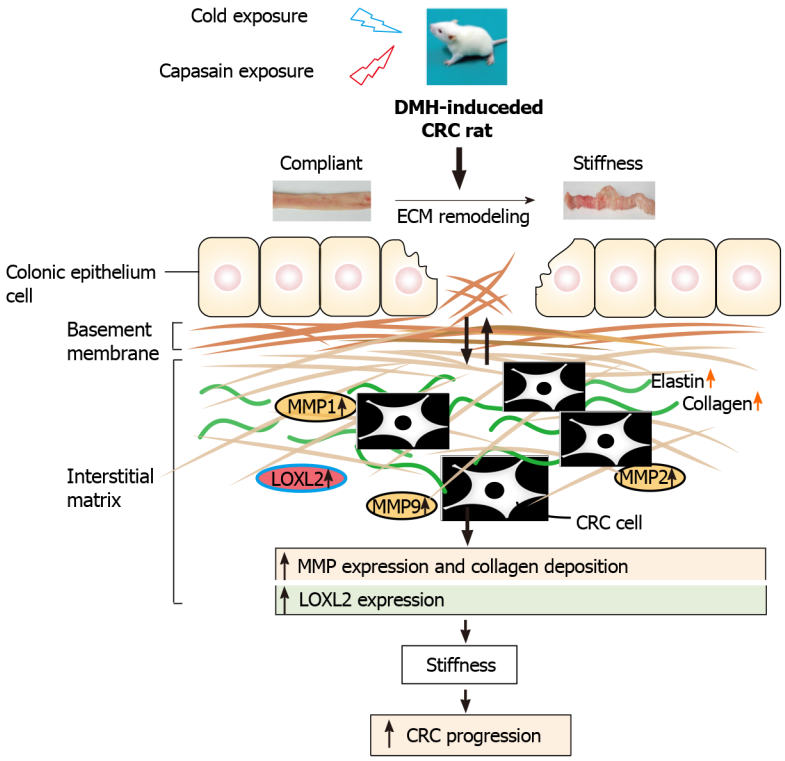

Figure 8 Schematic diagram depicting the role of extracellular matrix and extracellular matrix enzymes in promoting colorectal cancer pathogenesis. Comparison with the normal rats, rats exposed to cold and capsaicin with profound remodeling of extracellular matrix (ECM) in the colonic tissue, which was mediated by ECM enzymes. These results implicate a crucial role of ECM remodeling on cold and capsaicin exposure colorectal cancer development and progression. CRC: Colorectal cancer; DMH: 1,2-Dimethylhyrazine; MMP: Matrix metalloproteinase.

and they are also commonly expressed in tumor sites[52]. The expression of both MMP-2 MMP9 and TIMP-2 is higher in invasive tumors and is strongly associated with angiogenesis in DMH-induced CRC[24]. A previous study indicated that the cross-linking of collagen is known to activate enzymes involved in matrix remodeling, such as LOXL2, MMPs, and TIMPs[53,54]. MMPs are responsible for the degradation of ECM; LOXL2 mediate ECM cross-linking and stiffening[55]. However, recent studies indicated that LOXL2 activity promotes breast cancer metastasis by regulating the expression of MMPs and TIMPs involved in matrix remodeling[56]. LOXL2, TIMP1, and MMP9 are co-expressed during mammary metastasis, suggesting that they function together in glandular remodeling. Our previous study also found that expression levels of LOXL2, MMP1, MMP2, and MMP9 are positively correlated in CRC tissues, and they play synergistic roles in ECM remodeling of human CRC[37].

In the present study, the LOXL2, MMP1, MMP2, MMP9, and TIMP parameters were analyzed, and the results indicated a significant increase in the expression of these proteins in the cold exposure and capsaicin group accompanied by the enhancement of collagen and elastin deposition. Therefore, they may act together in regulating ECM remodeling. Growing insights from experimental studies on the roles of the ECM in CRC suggest that the quantitative and qualitative changes in ECM mediated by specific enzymes promote numerous cellular functions that steer cancer progression and metastasis[57,58]. In the present study, the ECM remodeling in the colonic tissue under cold and capsaicin exposure was more serious, thus increasing the exacerbation severity of CRC. Therefore, environmental factors, such as diet, will affect the internal and external constitutions of organism, causing different manifestations and disease progression in the organism. In the cold exposure and capsaicin treatment group, remodeling of the ECM and stromal stiffness is associated with increased propensity for progression to invasive CRC. Therefore, the levels of ECM remodeling can distinguish different organism characteristics and evaluate the CRC progression successfully, thus providing a novel pathological direction of analysis for clinicians. Furthermore, nanoscale mechanical imaging can be used to observe patients with heterogenous features of ECM, who would benefit most from ECM target therapies.

CONCLUSION

In summary, as shown in Figure 8, the present study revealed profound remodeling of the ECM in cold exposure and long-term administration of capsaicin at a low dose in rats. Collagen signatures including angle, length, width, and straightness have a great impact on CRC progression. Additionally, our results show that higher colonic tissue stiffness might result from ECM enzymes-mediated ECM crosslinking and excessive deposition of collagen and elastin, and such changes are strongly associated with the tumor progression of cold and capsaicin exposure CRC. A better understanding of the role of ECM remodeling and ECM enzymes on the pathogenic mechanisms of colon cancer may help in determining the molecular mechanism of CRC progression and could afford a novel therapeutic intervention in the treatment of this disease.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a cancer with high prevalence and mortality in the world. Extracellular matrix (ECM) is a dynamic compartment that regulates tissue development and homeostasis, and its remodeling contributes to neoplastic progression. The cancerous ECM can change cell phenotype and has profound influence on the colonization of metastatic cancer cells. However, the relationship between ECM remodeling and progression and aggression CRC from imposed by cold and capsaicin exposure remains unclear.

Research motivation

To identify the effect of cold exposure and capsaicin on ECM remodeling, ECM enzymes, and the underlying mechanism.

Research objectives

To explore the role of ECM remodeling and ECM enzymes in the 1,2-dimethylhydrazine (DMH)-induced CRC progression and the underlying mechanism.

Research methods

The CRC rat model was conducted by adding DMH and examining the role of ECM remodeling and ECM enzymes on DMH-induced CRC in the model. We investigated the morphology and structure of collagen and elastin using Masson's trichrome, Picrosirius red, and Weigert's Resorcin-Fuchsin stains. Additionally, we evaluated the protein expression level of type I collagen (COL I), type III collagen (COL III), elastin, lysyl oxidase-like 2 (LOXL2), matrix metalloproteinase (MMP) 1, MMP2, MMP9, and tissue-specific matrix metalloproteinase 1 by immunohistochemistry and observed the expression of COL I, COL III, elastin, and LOXL2 in the colon tissues of rats by reverse-transcriptase quantitative polymerase chain reaction.

Research results

We found that although there were no differences in the proportion of adenomas, a trend towards the increase of invasive tumors was observed in the cold and capsaicin group. Cold exposure group had a metastasis rate comparative with the other groups. Additionally, abnormal accumulation of both collagen and elastin was observed in the cold exposure and capsaicin group. Specifically, collagen quantitative analysis showed increased length, width, angle, and straightness compared with the DMH group. Collagen deposition and straightness were significantly increased in the cold exposure group compared with the capsaicin group. Cold exposure and capsaicin significantly increased the protein levels of COL I, elastin, and LOXL2 along with increases in their messenger RNA levels in the colon tissues compared with the DMH group, while COL III did not show a significant difference. Furthermore, in immunohistochemical evaluations, MMP1, MMP2, MMP9, and tissue-specific matrix metalloproteinase 1 staining increased in the cold exposure and capsaicin group compared with the DMH group.

Research conclusions

Increased stiffness of colonic tissue and the remodeling of ECM mediated by ECM enzymes resulted from cold and capsaicin exposure, predisposing an environment suitable for CRC development and progression.

Research perspectives

To target ECM in CRC tumor tissue could represent a novel potential therapeutic strategy.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Basic Study

Detection and analysis of common pathogenic germline mutations in Peutz-Jeghers syndrome

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Abstract

BACKGROUND

Different types of pathogenic mutations may produce different clinical phenotypes, but a correlation between Peutz-Jeghers syndrome (PJS) genotype and clinical phenotype has not been found. Not all patients with PJS have detectable mutations of the *STK11/LKB1* gene, what is the genetic basis of clinical phenotypic heterogeneity of PJS? Do PJS cases without *STK11/LKB1* mutations have other pathogenic genes? Those are clinical problems that perplex doctors.

AIM

The aim was to investigate the specific gene mutation of PJS, and the correlation between the genotype and clinical phenotype of PJS.

METHODS

A total of 24 patients with PJS admitted to the Air Force Medical Center, PLA (formerly the Air Force General Hospital, PLA) from November 1994 to January 2020 were randomly selected for inclusion in the study. One hundred thirty-nine common hereditary tumor-related genes including *STK11/LKB1* were screened and analyzed for pathogenic germline mutations by high-throughput next-generation sequencing (NGS). The mutation status of the genes and their relationship with clinical phenotypes of PJS were explored.

RESULTS

Twenty of the 24 PJS patients in this group (83.3%) had *STK11/LKB1* gene mutations, 90% of which were pathogenic mutations, and ten had new mutation sites. Pathogenic mutations in exon 7 of *STK11/LKB1* gene were significantly lower than in other exons. Truncation mutations are more common in exons 1 and 4 of *STK11/LKB1*, and their pathogenicity was significantly higher than that of missense mutations. We also found *SLX4* gene mutations in PJS patients.

CONCLUSION

PJS has a relatively complicated genetic background. Changes in the sites responsible for coding functional proteins in exon 1 and exon 4 of *STK11/LKB1* may be one of the main causes of PJS. Mutation of the *SLX4* gene may be a cause of genetic heterogeneity in PJS.

Key Words: Peutz-Jeghers syndrome; Genotype; Phenotype; STK11; Mutation

Core Tip: It is currently believed that Peutz-Jeghers syndrome (PJS) is an autosomal dominant genetic disease predominantly caused by germline mutations in the *STK11/LKB1* gene. No correlation of the PJS genotype and clinical phenotype has been found so far. The correlation of genotype and clinical phenotype and exploration of the internal molecular mechanism of different clinical phenotypes were studied in 24 treated PJS patients with different clinical phenotypes. Peripheral venous blood or normal tissue adjacent to polyps were collected for high-throughput next-generation sequencing (NGS) of 139 hereditary colorectal tumor-related genes including *STK11/LKB1*. A newly discovered likely pathogenic gene (*SLX4*) provided new data explaining the genetic heterogeneity of PJS.

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INTRODUCTION

It is currently believed that Peutz-Jeghers syndrome (PJS) is an autosomal dominant genetic disease predominantly caused by germline mutations in the *STK11/LKB1* gene. PJS is characterized by multiple hamartoma polyps in the gastrointestinal tract, pigmentation at specific sites, and hereditary tumors[1-4]. Pathogenic mutations of *STK11/LKB1* lead to inactivation of its expression product and loss of inhibition of mammalian target of rapamycin (mTOR) activity, which leads to abnormal activation of the LKB1/mTOR signal pathway and the occurrence of black spots on the skin and gastrointestinal hamartoma polyps[5]. More than 400 different pathogenic *STK11/LKB1* gene mutations are included in the Human Gene Mutation Database (HGMD), most of which are microminiature. Different types of pathogenic mutations may produce different clinical phenotypes, but no correlations of PJS genotype and clinical phenotype has been found so far[6], Not all patients with PJS have detectable mutations in the *STK11/LKB1* gene. What is the genetic basis of clinical phenotypic heterogeneity in PJS? Do PJS patients without *STK11/LKB1* mutations have other pathogenic genes? These are clinical problems that perplex doctors[7,8]. We enrolled 24 patients treated for PJS. Peripheral venous blood and normal tissue adjacent to polyps were collected for high-throughput next-generation sequencing (NGS) of 139 hereditary colorectal tumor-related genes including *STK11/LKB1* to study the correlation between genotype and clinical phenotype of PJS and explore the internal molecular mechanism of the clinical phenotypes.

patients (legal guardians of minors) understood the process and purpose of this study and signed an informed consent form. In the process of sample collection, follow the principles of informed consent in the Declaration of Helsinki, the Universal Declaration of Human Genome and Human Rights, and the Declaration of the Human Genome Ethics Committee on DNA Sampling, Control, and Acquisition. No additional data are available.

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MATERIALS AND METHODS

Study participants

Patients with PJS, from 18-70 years of age, met the clinical diagnostic criteria of PJs, had complete clinicopathological data, well preserved specimens, were eligible for inclusion. All participants gave their signed informed consent. Patients who could not provide experimental specimens or did not agree to participate in the study were excluded. Twenty-four PJS patients admitted to the Air Force Medical Center (formerly the Air Force General Hospital) from November 1994 to January 2020 met the above criteria and were enrolled. Their clinical information is shown in Table 1. Twenty-three were inpatients, one was an outpatient, 11 had family histories, and 12 had early onset pigment spots that had appeared when they were younger than 3 years of age. All patients met the PJS diagnostic criteria recommended by the National Comprehensive Cancer Network (NCCN)[9]. The experimental samples included 5 mL peripheral venous blood samples collected from 19 patients into tubes containing EDTA-2Na, and paraffin-embedded normal tissue surgically removed from areas adjacent to polyps in five patients. The study was reviewed and approved by the Ethics Committee of the Air Force Medical Center and the Second Affiliated Hospital of Zhejiang University School of Medicine. All patients or the legal guardians of minors, understood the process and purpose of this study and signed an informed consent form. Sample collection followed the ethical principles of the Declaration of Helsinki, the Universal Declaration of Human Genome and Human Rights, and the Declaration of the Human Genome Ethics Committee on DNA Sampling, Control, and Acquisition.

Methods

DNA was extracted from peripheral venous blood samples with TGuide Blood Genomic DNA Kits (CHI-TIANGEN) following the manufacturer's instructions. DNA was extracted from paraffin-embedded tissue specimens with QIAamp DNA FFPE micro sample tissue kits (GER-QIAGEN). Nucleic acids were broken into small, random 150-200 bp fragments by ultrasonic fragmentation (Covaris S220) and separated and evaluated with a Tapestation 2200 electrophoresis working platform (Agilent) to check whether the fragments met the requirements for library construction. A standard gene library was constructed using KAPA HyperPlus Kit (Illumina). A panel of 139 common tumor genetic susceptibility genes including colorectal cancer (Table 2) was selected and provided by Genetron Health Co.(Beijing). The specific gene capture probe was hybridized with the library in the environment of a hybridization buffer, and purified by the magnetic bead method. High-throughput NGS was performed with a Novaseq 6000 sequencer (Illumina, United States). Trimmomatic (version 0.33) was used to crop and filter the original data, which was stored in FastQ format, after sequencing. The reads at the end of each pair were aligned with the human reference sequence GRCh37 (hg19) using the BWA-MEM algorithm (BWA version 0.7.10-r789) and the default parameters. The Picard tool (version 1.103 http://broadinstitute.github.io/picard/) was used to delete duplicate readings, and GATK (version 3.1-0-g72492bb) was used to realign the sequences around the known insertion loss at the single sample level and to recalibrate the base quality. Integrative Genomics Viewer version 2.3.34 (https://software.broadinstitute.org/software/igv/) was used to check the mutations in the coding region.

The Chinese (1000 CN), general population (1000 MAF). and dbSNP (https://www.ncbi.nlm.nih.gov/) at 1000 Genome Project (http://ftp.ncbi.nih.gov/) Snip/), ESP6500 AA/EA (NHLBI GO Exome Sequencing Project https://evs gs.washington.edu/EVS/), ExAC MAF (The Exome Aggregation Consortium) and other population databases were searched for the mutation frequency of this gene. The location of genes with a mutation frequency < 0.01 in the HGMD database (HGMD-PUBLIC version 20152) were used for pathogenicity analysis.

The diseases that the variant gene was related to were searched in the OMIM disease database (https://omim.org/) by ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). HGMD https://www.hgmd.cf.ac.uk) retrieved the description of the mutation. SIFT[10] (http://sift.jcvi.org), PolyPhen2[11] (http://genetics.bwh.harvard.edu/pph2), and Mutation Assessor (http://mutationassessor.org) make conservative predictions of amino acid sequences. The results were used to evaluate the pathogenicity of the mutations[12,13].

SPSS 24.0 was used for statistical analysis of the acquired data. Qualitative results were reported as numbers and percentages. The chi-square test or Fisher's exact probability method was used for between-group comparisons. $P < 0.05$ was considered

Table 1 Clinical characteristics of 24 enrolled Peutz-Jeghers syndrome patients

No.	Gender	Specimen	Time since onset of pigment spots (yr)	Early or late onset	Family history (members)	Number of hospitalizations	Number of operations	Stomach and enteroscopy times	Age at initial diagnosis of polyps	Age at first treatment	Polyp pathology	Load of Gastric polyps/Max. diameter (mm)	Load of small intestinal polyps/Max. diameter (mm)	Load of colorectal polyps/Max. diameter (mm)
1	Male	Paraffin section	20	Late	No	2	1	6	20	15	1	/	20/30	/
2	Male	Paraffin section	6	Late	Yes (mother and sister)	1	2	3	9	9	1	2/16	20/40	1/8
3	Female	Paraffin section	4	Late	No	2	1	4	9	9	1	/	3/28	/
4	Male	Paraffin section	5	Late	No	1	2	1	21	21	3	20/4	6/50	/
5	Male	Paraffin section	1	Early	Yes (mother)	4	2	1	4	4	1	2/12	2/60	/
6	Female	Blood	5	Late	Yes (father)	1	0	1	29	29	1	/	/	/
7	Female	Blood	1	Early	Yes (father and sister)	4	0	11	7	7	1	1/8	2/30	3/40
8	Male	Blood	0	Early	Yes (father and sister)	1	0	1	10	10	1	/	10/50	/
9	Male	Blood	6	Late	Yes (mother and grandmother)	4	1	7	6	7	1	5/12	2/30	3/35
10	Female	Blood	2	Early	No	1	0	3	7	7	1	2/15	/	1/30
11	Male	Blood	3	Late	No	1	4	0	22	32	1	/	1/30	/
12	Male	Blood	2	Early	No	2	1	10	4	4	1	1/6	2/50	/
13	Male	Blood	2	Early	No	1	2	1	25	24	1	/	10/20	/
14	Female	Blood	3	Late	No	8	2	8	6	6	1	1/10	8/80	1/20
15	Male	Blood	5	Late	No	1	2	3	20	19	2	1/6	1/80	2/30
16	Male	Blood	1	Early	Yes (mother)	3	0	2	10	9	1	/	1/25	/
17	Male	Blood	1	Early	No	3	1	4	6	6	1	8/40	10/30	/
18	Female	Blood	1	Early	No	6	2	9	11	10	1	1/15	3/35	1/50
19	Female	Blood	3	Late	Yes (mother)	2	0	4	15	15	1	1/12	2/12	1/25

20	Female	Blood	3	Late	Yes (father, uncle, and grandmother)	2	2	5	7	7	1	/	18/50	/
21	Female	Blood	1	Early	Yes (mother, uncle, and aunt)	2	0	4	31	31	1	/	10/50	10/40
22	Female	Blood	2	Early	Yes (father and brother)	1	0	1	6	6	1	10/10	8/50	/
23	Male	Blood	5	Late	No	1	0	2	11	11	1	1/30	5/70	1/30
24	Male	Blood	2	Early	No	1	0	4	5	4	1	10/15	/	/

(1) *STK11* mutation, *SLX4* mutation, other gene mutation groups: 0: None 1: Yes; (2) Early onset: Pigment spots appeared at < 3 years of age; Late onset: Pigment spots appeared at ≥ 3 years of age; (3) Polyp pathology: 1 hamartoma, 2 hamartoma with adenoma, 3 hamartoma with cancer; (4) Polyp load is the number of polyps, the largest diameter unit is mm; and (5) 6 was an outpatient, the results of previous endoscopy are unknown.

statistically significant.

RESULTS

STK11/LKB1 gene detection results and pathogenicity analysis

Twenty of the 24 PJS patients (83.3%) in this group had *STK11/LKB1* gene mutations (Table 3). All were heterozygous and ten were newly discovered mutation sites not included in the dbSNP database. There were eight frameshift mutations, five splice-site mutations, four missense mutations and three nonsense mutations. The mutations occurred in eight of the ten exons in the *STK11/LKB1* gene, mutations in exons 1 and 4 and 4 each in exon 7, two in each exons 5 and 8, and one in exons 2, 3, and 6. Frameshift mutations, splice-site mutations, and nonsense mutations were all related to pathogenicity. Frameshift mutations accounted for 62.5% (5/8) that were clearly pathogenic, and 37.5% (3/8) that might cause disease. Splice-site mutations accounted for 40% (2/5) that are clearly pathogenic, and 60% (3/5) that might cause disease. All three nonsense mutations were clearly pathogenic, and the missense mutations were related to and might cause disease. Sites of unclear clinical significance accounted for 50% (2/4); of the 11 truncated mutations, eight cases were clearly pathogenic and three were likely to cause disease. The pathogenicity of STK11 gene mutations in exon 7 was significantly lower than that of other exons (P = 0.000). Truncation mutations were significantly more pathogenic than missense mutations (P = 0.012). The prediction results of bioinformatics tools for missense mutations are shown in Table 4, and the relevant database records and the pathogenicity judgment of all mutations are shown in Table 5.

Table 2 Cancer genetic susceptibility 139 gene panel coverage

AIP	*CYLD*	*FANCL*	*MLH3*	*PRSS1*	*SMARCA4*
ALK	*DDB2*	*FANCM*	*MRE11A*	*PTCH1*	*SMARCB1*
APC	*DICER1*	*FAS*	*MSH2*	*PTCH2*	*SMARCE1*
ATM	*DIS3L2*	*FH*	*MSH6*	*PTEN*	*SOS1*
ATR	*EGFR*	*FLCN*	*MTAP*	*PTPN11*	*STAT3*
AXIN2	*ELANE*	*GALNT12*	*MTUS1*	*RAD50*	*STK11*
BAP1	*EPCAM*	*GATA2*	*MUTYH*	*RAD51B*	*SUFU*
BARD1	*ERCC1*	*GEN1*	*NBN*	*RAD51C*	*TERT*
BLM	*ERCC2*	*GJB2*	*NF1*	*RAD51D*	*TGFBR1*
BMPR1A	*ERCC3*	*GPC3*	*NF2*	*RB1*	*TMEM127*
BRCA1	*ERCC4*	*GREM1*	*NSD1*	*RECQL*	*TP53*
BRCA2	*ERCC5*	*HMBS*	*NTRK1*	*RECQL4*	*TSC1*
BRIP1	*EXT1*	*HNF1A*	*PALB2*	*RET*	*TSC2*
BUB1B	*EXT2*	*HOXB13*	*PALLD*	*RHBDF2*	*UROD*
CBL	*EZH2*	*HRAS*	*PDGFRA*	*RUNX1*	*USHBP1*
CDC73	*FANCA*	*KIT*	*PHOX2B*	*SBDS*	*VEGFA*
CDH1	*FANCB*	*LASP1*	*PMS1*	*SDHA*	*VHL*
CDK4	*FANCC*	*MAX*	*PMS2*	*SDHAF2*	*WRN*
CDKN1B	*FANCD2*	*MC1R*	*POLD1*	*SDHB*	*WT1*
CDKN1C	*FANCE*	*MEN1*	*POLE*	*SDHC*	*XPA*
CDKN2A	*FANCF*	*MET*	*POLH*	*SDHD*	*XPC*
CEBPA	*FANCG*	*MTTF*	*PPM1D*	*SLX4*	*XRCC2*
CHEK1	*FANCI*	*MLH1*	*PRKAR1A*	*SMAD4*	*ZMAT3*
CHEK2					

Considering that the type of specimen may impact on the detection rate of *STK11/LKB1* gene mutations, we analyzed the paraffin-embedded tissue and blood samples separately. The detection rate of *STK11/LKB1* mutations in 60 patients with paraffin samples was 60% (3/5), slightly less than the 89.4% (17/19) of the blood samples from 19 patients. The difference in mutation detection rate of this gene in the two types of sample was not statistically different (P = 0.116).

SLX4 gene detection results and pathogenicity analysis

SLX4 gene mutation (Table 6) was detected in 5 PJS patient samples in this group, with a total detection rate of 20.83% (5/24), all of which were heterozygous mutations. The mutation occurred in 4 of 15 exons of *SLX4* gene. Mutation types include: 3 missense mutations, one splice-site mutation, and one non-frameshift mutation. No truncation mutation was found. The *SLX4* gene is a tumor suppressor gene, and there are three newly discovered mutation sites. The prediction results of three cases of missense mutations by bioinformatics tools (Table 7), the collection of relevant databases and the judgment of the pathogenicity of all mutations (Table 8) are as follows.

Other gene detection results and pathogenicity analysis

A total of 55 mutations of 46 genes other than *STK11/LKB1* and *SLX4* were detected in 21 cases (Table 9), f a detection rate of 87.5% (21/24). Twenty-three of the genes were related to cancer suppression and had 32 different mutation sites. Two mismatch repair *MMR* genes were detected, *MSH2*, *MSH6*. Except for a frameshift mutation (frameshift deletion) in the *BRIP1* gene detected in one patient (No. 18), the rest were missense mutations (Table 10).

Table 3 Characteristics of *STK11/LKB1* gene mutations

No.	Mutation type	dbSNP RS	Mutation site	Amino acid change	Exon	Variant type
2	Frameshift	rs372511774	c.357delC	p.N119Kfs	2|10	SNV
4	Splice-site variant	rs398123406	c.921-1G>A	/	8|10	SNP
5	Frameshift	rs1060499961	c.131dupA	p.L45Afs	1|10	INS
6	Missense	/	c.869T>C	p.L290P	7|10	SNP
7	Nonsense	/	c.658C>T	p.Q220X	5|10	SNP
8	Frameshift	/	c.548del	p.L183Rfs	4|10	DEL
9	Splice-site variant	rs398123406	c.921-1G>C	/	8|10	SNP
10	Frameshift	/	c.471_472del	p.F157Lfs	4|10	DEL
12	Frameshift	/	c.180del	p.Y60X	1|10	DEL
13	Missense	/	c.869T>A	p.L290H	7|10	SNP
14	Splice-site variant	/	c.598-2A>G	/	5|10	SNP
15	Missense	rs121913315	c.580G>A	p.D194N	4|10	SNP
16	Missense	rs730881978	c.890G>A	p.R297K	7|10	SNP
17	Frameshift	/	c.577_578del	p.S193Rfs	4|10	DEL
18	Splice-site variant	/	c.863-2A>G	/	7|10	SNP
19	Splice-site variant	rs1555735080	c.290+1G>T	/	1|10	SNP
20	Nonsense	/	c.179dup	p.Y60X	1|10	INS
21	Frameshift	rs587782584	c.842dup	p.L282Afs	6|10	INS
23	Frameshift	rs786203886	c.228dup	p.V77Rfs	1|10	INS
24	Nonsense	rs730881970	c.409C>T	p.Q137X	3|10	SNP

DEL; Deletion; INS: Insertion; SNP: Single nucleotide polymorphism; SNV: Single nucleotide variation.

Table 4 Prediction of protein function change caused by *STK11/LKB1* mutation

No.	PolyPhen		Mutation Assessor		SIFT	
	Score	Prediction	Score	Prediction	Score	Prediction
6	1	Probably damaging	0.98351; 4.21	High	0	Deleterious
13	1	Probably damaging	0.99415; 4.555	High	0	Deleterious
15	1	Probably damaging	0.98178; 4.165	High	0	Deleterious
16	1	Probably damaging	0.98818; 4.34	High	0.01	Deleterious
23	0.022	Benign	0.56769; 1.78	Low	0.26	Tolerated

STK11/LKB1 genotype-phenotype correlation analysis

Investigation of the relationship between genotype and family history found that the proportion of patients with truncated mutations was slightly higher in those with a family history than in those without a history (60% *vs* 50%). The proportion of splice-site mutations was lower in those with a family history (20% *vs* 30%), and the proportion of nonsense mutations was higher in patients with a family history (20.0% *vs* 11.1%). The proportions of missense mutations were the same (20% *vs* 20%), and the proportion of frameshift mutations were also equal (40% *vs* 10%). There were no significant difference between-group differences in $P_{truncation\ mutation} = 0.653$, $P_{splice\ site\ mutation} = 0.606$, $P_{nonsense\ mutation} = 0.371$, $P_{missense\ mutation} = 1.000$, and $P_{frameshift\ mutation} = 1.000$.

Evaluation of the relationship between genotype and early onset/late onset found that the proportion of truncated mutations in patients with early onset was higher than that in patients with late onset (72.7% *vs* 33.3%). In patients with early onset, the

Table 5 *STK11/LKB1* mutation-related databases and pathogenicity analysis

No.	cDNA/protein	Disease database			Pathogenic judgment
		HGMD	ClinVar	OMIM	
2	p.N119Kfs	/	(1/1) pathogenic	/	Pathogenic
4	c.921-1G>A	√	/	PJS	Pathogenic
5	p.L45Afs	/	/	/	Pathogenic
6	p.L290P	√	(1/1) pathogenic	PJS	Clinical significance unknown
7	p.Q220X	/	(3/3) pathogenic	PJS	Pathogenic
8	p.L183Rfs	/	/	PJS	Pathogenic
9	c.921-1G>C	√	(2/2) pathogenic	PJS	Pathogenic
10	p.F157Lfs	√	/	PJS	Likely pathogenic
12	p.Y60X	√	√	PJS	Pathogenic
13	p.L290H	/	/	PJS	Clinical significance unknown
14	c.598-2A>G	/	(1/1) pathogenic	PJS	Likely pathogenic
15	p.D194N	√	(4/6) likely pathogenic; (2/6) pathogenic	PJS	Likely pathogenic
16	p.R297K	√	(1/2) pathogenic; (1/2) unknown	PJS	Likely pathogenic
17	p.S193Rfs	/	/	PJS	Likely pathogenic
18	c.863-2A>G	/	(1/1) pathogenic	PJS	Likely pathogenic
19	c.290+1G>T	Pathogenic	/	PJS	Likely pathogenic
20	p.Y60X	Pathogenic	(2/2) pathogenic	PJS	Pathogenic
21	p.L282Afs	Pathogenic	(1/1) pathogenic	PJS	Pathogenic
23	p.V77Rfs	/	/	PJS	Likely pathogenic
24	p.Q137X	Pathogenic	(1/1) pathogenic	PJS	Pathogenic

(4/6) likely pathogenic: A total of six institutions have judged this mutation, four of which are judged as probably pathogenic, the same below. PJS: Peutz-Jeghers syndrome.

Table 6 Characteristics of *SLX4* gene mutations

No.	Mutation type	dbSNP RS	Mutation site	Amino acid changes	Exon	Variant type
1	Missense	rs551385115	c.5072A>G	p.N1691S	14|15	SNP
2	Splice-site variant	/	c.1683+1G>A	splice	7|15	SNP
3	Missense	rs774243118	c.2990C>T	p.P997L	12|15	SNP
18	Missense	/	c.2425G>C	p.E809Q	12|15	SNP
22	Non-frameshift	/	c.568_570del	p.P190del	3|15	DEL

DEL: Deletion; SNP: Single nucleotide polymorphism.

percentages of frameshift mutations (54.5% *vs* 22.2%) and sense mutations (18.2% *vs* 11.1%) were higher than those in late onset patients. The percentages of splice-site mutations (9% *vs* 44.4%) and missense mutations were lower (18.2% *vs* 22.2%). There were no significant between-group differences in $P_{truncation\ mutation} = 0.078$, $P_{frameshift\ mutation} = 0.142$, $P_{nonsense\ mutation} = 0.660$, $P_{splice\ site\ mutation} = 0.069$, $P_{missense\ mutation} = 0.822$.

DISCUSSION

The *STK11/LKB1* gene located on chromosome 19p13.3 is considered to be a tumor

Table 7 Prediction of protein function change caused by *SLX4* mutation

No.	PolyPhen		Mutation assessor		SIFT	
	Score	Prediction	Score	Prediction	Score	Prediction
1	0	Benign	0.08118; 0	Neutral	0.16	Tolerated
3	0.004	Benign	0.05510; -0.035	Neutral	1	Tolerated /
18	0.341	Benign	0.59436; 1.845	Low	0.04	Deleterious

Table 8 *SLX4* mutation-related databases and pathogenicity analysis

No.	cDNA/Protein	Disease database			Pathogenic judgment
		HGMD	ClinVar	OMIM	
1	p.N1691S	/	(1/1)Uncertain Significance	BTB/POZ domain containing 12*SLX4* structure-specific	Clinical significance unknown
2	c.1683+1G>A	/	/	BTB/POZ domain containing 12\SLX4 structure-specific	Likely pathogenic
3	p.P997L	/	/	BTB/POZ domain containing 12\SLX4 structure-specific	Clinical significance unknown
18	p.E809Q	√	/	BTB (POZ) domain containing 12\SLX4 structure-specific	Clinical significance unknown
22	p.P190del	/	/	BTB (POZ) domain containing 12\SLX4 structure-specific	Clinical significance unknown

suppressor gene[14] and is widely expressed in human tissues. Pathogenic mutation of *STK11* can inactivate its expressed product, which results in the loss of its inhibitory effect on the activity of mammalian target of rapamycin (mTOR), leading to the occurrence of skin and mucous membrane black spots and gastrointestinal polyps[5]. Methylation of the *STK11/LKB1* gene promoter has an important role in the process of malignant transformation of gastrointestinal polyps[15]. At present, the comprehensive mutation rate of *STK11/LKB1* gene in PJS patients detected by multiple sequencing methods is about 80%-94%[8,15,16]. The detection rate of *STK11/LKB1* gene mutation in PJS patients in this study was 83.3% (20/24), 90% of which are related to pathogenicity. Analysis of the pathogenicity of all the detected mutation sites included in the Mendelian Inheritance in Man (OMIM) database found that about 90% of the *STK11/LKB1* mutations were related to PJS. Except for the *STK11/LKB1* gene and one case of *SLX4* gene mutation, no other gene mutations related to the disease or the possibility of disease were found.

Research on whether there is a correlation between the PJS genotype and clinical phenotype is ongoing. Although the correlation is currently unclear[6,17], some studies have reported positive results. For example, Forcet *et al*[18] reported that patients often present with only black spots and without gastrointestinal polyps when heterozygous mutations occur in exon 8 of the *STK11* gene. Amos *et al*[19] found that PJS patients with missense mutations had a first episode of polypectomy and appearance of other symptoms significantly later than those with truncated mutations or no detectable mutations. In a study including 116 PJS patients in 52 families, Wang *et al*[20] found that nearly 30% of the mutations occurred in exon 7, and some of those mutations affected the protein Kinase domain XI region, which is associated with 90% of cases with gastrointestinal polyp dysplasia. An analysis of the start region of the *STK11/LKB1* coding sequence by Hearle *et al*[21] found that a change in promoter sequence was unlikely to be the cause of PJS. In this study the time that dark spots first appeared, which is a relatively objective indicator, was the basis of clinical classification, and was used to determine whether there was a correlation between the appearance of the spots and any of the genotypes. Spots that appear in early childhood will be noticed. On the other hand, unless there are obvious clinical symptoms, it is extremely difficult to know about gastrointestinal polyps that appear in early childhood. Also, PJS is an autosomal dominant genetic disease and does not completely follow Mendelian inheritance[6]. In clinical practice, it is often found that neither parent has a family history but their child has the disease. This is difficult to fully explain if the disease is caused by a single gene. Therefore, whether the patient has a family history was also included in the basis of clinical classification.

This study did not found that patients with different clinical phenotypes (early onset/late onset and with or without a family history) had statistically significant differences in their *STK11/LKB1* gene mutations and loci. However, we found that the

Table 9 Other gene mutations and inclusion in relevant database

No.	Gene	Type	Mutation site	Amino acid changes	Exon	Disease database		
						HGMD	ClinVar	OMIM
1	*BARD1*	TSG	c.556A>G	p.S186G	4|11	/	(6/6)Uncertain Significance	/
	EGFR	/	c.61G>A	p.A21T	1|28	/	/	Epidermal growth factor receptor
2	*GEN1*	/	c.181T>A	p.S61T	3|14	/	/	Gen endonuclease homolog 1
	BRCA1	TSG	c.2387C>T	p.T796I	10|23	/	(8/8)Uncertain Significance	/
4	*NTRK1*	/	c.1604A>G	p.E535G	13|17	/	/	/
	PDGFRA	/	c.1423G>A	p.E475K	10|23	/	/	/
	TSC2	TSG	c.521C>T	p.S174L	6|42	/	(2/2)Uncertain Significance	/
	MSH6	/	c.1063G>A	p.G355S	4|10		(4/7)Uncertain Significance(3/7)likely benign	/
5	*EGFR*	/	c.3040G>A	p.D1014N	25|28	/	/	Epidermal growth factor receptor
	MTUS1	TSG	c.2282G>A	p.S761N	3|15	/	/	Mitochondrial tumor suppressor 1
	PTCH1	TSG	c.2222C>T	p.A741V	14|24	/	(3/4)benign, (1/4)likely benign	/
6	*SDHA*	TSG	c.715A>G	p.I239V	6|15	√	(2/2)Uncertain significance	/
	MTUS1	TSG	c.1866C>G	p.N622K	2|15	√	√	Mitochondrial tumor suppressor 1
7	*RECQL4*	/	c.1048A>G	p.R350G	5|21	/	(1/1)Uncertain Significance	/
	RECQL4	/	c.236G>A	p.G79E	4|21	/	/	/
8	*ATM*	TSG	c.6503C>T	p.S2168L	45|63	/	(7/7)Uncertain Significance	Ataxia telangiectasia mutated
10	*TSC2*	TSG	c.3475C>T	p.R1159W	30|42	/	(2/4)benign, (2/4)likely benign	/
	FANCG	TSG	c.458C>G	p.A153G	4|14	/	(1/1)Uncertain Significance	/
11	*SBDS*	/	c.98A>G	p.K33R	1|5	/	/	/
12	*VHL*	TSG	c.134C>T	p.P45L	1|3	/	/	Von Hippel-Lindau syndrome
	FANCA	/	c.3031C>T	p.R1011C	31|43	/	(1/1)likely benign	/
	TP53	TSG	c.620A>G	p.D207G	6|11	√	/	/
13	*FANCA*	/	c.2944A>G	p.T982A	30|43	/	(2/2)Uncertain Significance	/
14	*PALLD*	/	c.1011C>A	p.D337E	3|21	/	/	/
	MLH3	TSG	c.1519A>G	p.M507V	2|13	/	(1/1)Uncertain Significance	Mutl (E. Coli) homolog 3
	SMARCA4	TSG	c.3791C>T	p.T1264M	28|36	/	(3/3)Uncertain Significance	/
	NF1	TSG	c.3940T>C	p.W1314R	29|58	/	(1/1)Uncertain Significance	/
15	*PTCH1*	TSG	c.2222C>T	p.A741V	14|24	/	(1/1)likely benign	/
	GALNT12	/	c.148C>A	p.P50T	1|10	/	/	/
16	*ATR*	TSG	c.325C>T	p.R109W	4|47	/	(1/1)Uncertain Significance	Ataxia telangiectasia and Rad3 related
	VEGFA	TSG	c.1039G>A	p.V347I	6|8	/	/	Vascular endothelial growth factor
	DIS3L2	/	c.1642G>A	p.A548T	13|21	/	/	/
17	*TSC1*	TSG	c.2693C>G	p.T898S	21|23	√	(3/5)likely benign, (1/5)benign, (1/5)Uncertain significance	/
18	*PTCH1*	TSG	c.109G>T	p.G37W	1|24	√	(1/1)Uncertain Significance	/

	BRIP1	/	c.3072del	p.S1025Hfs	20|20	√	(1/2)likely pathogenic, (1/2)Uncertain significance	/
	WRN	/	c.3778G>A	p.A1260T	32|35	/	(2/2)Uncertain significance	werner syndrome
	RECQL	/	c.166G>A	p.G56R	4|16	/	/	/
19	*BARD1*	TSG	c.1148T>G	p.M383R	4|11	/	/	/
	USHBP1	/	c.1358C>T	p.P453L	9|13	/	/	/
	APC	TSG	c.2882A>G	p.N961S	16|16	/	(1/1)Uncertain Significance	Adenomatosis polyposis coli
20	*DICER1*	TSG	c.2113A>G	p.I705V	13|27	/	/	Multinodular goiter
	FANCM	/	c.2762G>A	p.C921Y	14|23	/	/	/
	APC	TSG	c.5257G>C	p.A1753P	16|16	/	(3/3)Uncertain Significance	Adenomatosis polyposis coli
	NSD1	/	c.5493T>G	p.D1831E	16|23	/	/	Sotos syndrome
	SDHA	TSG	c.739A>G	p.I247V	6|15	/	(4/4)Uncertain Significance	/
	MTUS1	TSG	c.908A>G	p.N303S	2|15	/	/	Mitochondrial tumor suppressor 1
22	*EXT2*	TSG	c.896G>A	p.R299H	5|14	√	(1/2)likely benign, (1/2)uncategorized	/
	ATM	TSG	c.1555G>A	p.V519I	10|63	√	(3/3)Uncertain Significance	Ataxia telangiectasia mutated
	BRCA2	TSG	c.1568A>G	p.H523R	10|27	√	(1/12)benign, (9/12)likely benign, (2/12)Uncertain Significance	Fanconi anemia
	TP53	TSG	c.214C>G	p.P72A	4|11	√	(5/5)Uncertain Significance	/
23	*FLCN*	TSG	c.1366G>C	p.D456H	12|14	/	/	
	MSH2	TSG	c.1789G>A	p.D597N	12|16	/	(1/1)Uncertain Significance	Colon cancer, nonpolyposis type 1
	KIT	/	c.2263G>A	p.A755T	16|21	/	(1/2)Uncertain Significance,(1/2)uncategorized	Piebald trait
24	*BAP1*	TSG	c.1154G>A	p.R385Q	12|17	/	(2/2)Uncertain Significance	/
	TSC2	TSG	c.1609C>T	p.R537C	16|42	√	(1/5)benign, (2/5)likely benign; (1/5)Uncertain Significance; (1/5)uncategorized	/

HGMD: Human Gene Mutation Database; OMIM: Online Mendelian Inheritance in Man; TSG: Tumor suppressor gene.

most truncation mutations of the *STK11/LKB1* gene mostly occurred in exons 1 and 4, most missense mutations occurred in exon 7, and that truncation mutations were significantly more pathogenic than missense mutations. The results indicate that changes in the sites encoding functional proteins in exon regions 1 and 4 may be among the main causes of PJS. Also, the percentage of *STK11/LKB1* truncation mutations in patients with early onset PJS was higher than that in patients with late onset PJS, and the between-group difference in the percentage of missense mutations was not significant. Because the evidence of a correlation with missense mutations was not strong, it suggests that early onset PJS is more likely to be caused by pathogenic mutations in *STK11/LKB1*, while late onset disease is likely to be clinically heterogeneous. The study results also suggest that analysis of the age of appearance of dark spots in a large sample of PJS patients would yield some interesting findings.

For the first time, we detected more concentrated mutations in the *SLX4* gene in PJS patients. The *SLX4* (*FANCP*) gene is a tumor suppressor gene located on chromosome 16p13.3[21]. It serves as a key scaffold element for the assembly of multiprotein complexes containing enzymes involved in DNA maintenance and repair[22] and has low to moderate expression in all adult and fetal tissues and specific adult brain regions[23]. It has been reported that[24] truncated mutations in the *SLX4* gene were detected in families with Fanconi anemia, and it was determined that *SLX4* mutations are clearly related to one of the subtypes of the disease. Fanconi anemia is a rare autosomal recessive genetic disease[25]. In addition to blood system-related manifestations, the clinical manifestations of FA include multiple congenital malformations, brown pigmentation of the skin, and tumor susceptibility[26]. There are many similarities with PJS, mutations in the *SLX4* gene have been detected in patients with PJS in previous studies, the first of which was found in this group. *SLX4* is considered

Table 10 Prediction of protein function changes caused by other gene mutations

Gene	SIFT		PolyPhen		Mutation Assessor	
	Score	Prediction	Score	Prediction	Score	Prediction
BARD1	0	Deleterious	0.144	Benign	0.66939; 2.045	Medium
EGFR	0.4	Tolerated	0.956	Probably damaging	0.33485; 1.01	Low
GEN1	0	Deleterious	0.999	Probably damaging	0.34521; 1.04	Low
BRCA1	0.02	Deleterious	0.775	Probably damaging	0.78223; 2.4	Medium
NTRK1	0.01	Deleterious	0.639	Probably damaging	0.02685; -0.53	Neutral
PDGFRA	0.1	Tolerated	0.05	Benign	0.38838; 1.175	Low
TSC2	0.15	Tolerated	0.327	Benign	0.57536; 1.79	Low
MSH6	0.45	Tolerated	0.176	Benign	0.08118; 0	Neutral
EGFR	0	Deleterious	0.814	Possibly damaging	0.83953; 2.67	Medium
MTUS1	0.09	Tolerated	0.044	Benign	0.27053; 0.805	Low
PTCH1	0	Deleterious	0.7	Possibly damaging	0.88377; 2.95	Medium
SDHA	0.01	Deleterious low confidence	0.078	Benign	0.49699; 1.58	Low
MTUS1	0.01	Deleterious	0.096	Benign	0.29908; 0.895	Low
RECQL4	/	/	/	/	/	/
RECQL4	/	/	/	/	/	/
ATM	0	Deleterious	0.294	Benign	0.67953; 2.075	Medium
TSC2	0.01	Deleterious	0.226	Benign	0.08118; 0	Neutral
FANCG	0.03	Deleterious	0.018	Benign	0.14661; 0.345	Neutral
SBDS	0.12	Tolerated	0.051	Benign	0.71920; 2.185	Medium
VHL	0.06	Tolerated	0.012	Benign	0.19112; 0.55	Neutral
FANCA	0.24	Tolerated	0	Benign	0.02315; -0.6	Neutral
TP53	0.03	Deleterious	0.386	Benign	0.45228; 1.405	Low
FANCA	0.79	Tolerated	0.007	Benign	0.52573; 1.65	Low
PALLD	0.7	Tolerated	0.159	Benign	0.00602; -1.34	Neutral
MLH3	0.47	Tolerated	0	Benign	0.55103; 1.725	Low
SMARCA4	0.05	Deleterious	0.007	Benign	0.29908; 0.895	Low
NF1	0.62	Tolerated	0.015	Benign	0.08118; 0	Neutral
PTCH1	0	Deleterious	0.626	Possibly damaging	0.88377; 2.95	Medium
GALNT12	0.11	Tolerated	0.007	Benign	0.51422; 1.61	Low
ATR	0	Deleterious	0.998	Probably damaging	0.65975; 2.015	Medium
VEGFA	0.25	Tolerated low confidence	0.695	Probably damaging	0.08118; 0	Neutral
DIS3L2	0.05	Tolerated	0.996	Probably damaging	0.87328; 2.875	Medium
TSC1	/	/		/	0.00621; -1.32	Neutral
PTCH1	0.03	Deleterious low confidence	0.259	Benign	0.36672; 1.1	Low
BRIP1	/	/	/	/	/	/
WRN	0.59	Tolerated	0.164	Benign	0.70595; 2.14	Medium
RECQL	0.5	Tolerated	0.005	Benign	0.41079; 1.255	Low
BARD1	0.4	Tolerated	0	Benign	0.08118; 0	Neutral
USHBP1	0.05	Tolerated	0.521	Possibly damaging	0.56769; 1.78	Low
APC	0.16	Tolerated	0.82	Possibly damaging	0.46157; 1.445	Low

DICER1	0.29	Tolerated	0.664	Possibly damaging	0.34521; 1.04	Low
FANCM	1	Tolerated	0	Benign	0.40543; 1.245	Low
APC	0.57	Tolerated low confidence	0.003	Benign	0.14661; 0.345	Neutral
NSD1	0.03	Deleterious	0.684	Possibly damaging	0.66939; 2.045	Medium
SDHA	0.02	Deleterious low confidence	0.02	Benign	0.20574; 0.59	Neutral
MTUS1	0.87	Tolerated	0	Benign	0.12746; 0.255	Neutral
EXT2	0.03	Deleterious	0.993	Possibly damaging	0.82323; 2.585	Medium
ATM	0.58	Tolerated	0.007	Benign	0.56769; 1.78	Low
BRCA2	0.09	Tolerated	0.003	Benign	0.08118; 0	Neutral
TP53	0.94	Tolerated	0	Benign	0.03608; -0.345	Neutral
FLCN	0.03	Deleterious	0	Benign	0.47716; 1.5	Low
MSH2	0.25	Tolerated	0.023	Benign	0.39692;1.235	Low
KIT	0.15	Tolerated	0.472	Possibly damaging	0.03608; -0.345	Neutral
BAP1	0	Deleterious low confidence	0.968	Possibly damaging	0.59436; 1.845	Low
TSC2	0.02	Deleterious	0.446	Possibly damaging	0.75777; 2.31	Medium

to be an important regulator of DNA repair. Studies have shown that repairing specific types of DNA damage requires *SLX4* and other endonucleases to participate together [22]. At present, it is believed that[27-29] the loss of DNA MMR genes causes the accumulation of mismatches in the process of DNA replication, resulting in the occurrence of microsatellite instability and partial junctions. Colorectal cancer has obvious genetic characteristics. We also detected mutations in some MMR genes (*MSH2* and *MSH6*) in PJS, and the role of *SLX4* gene is highly similar to that. Perhaps the mutation of the *SLX4* gene may explain the genetic heterogeneity of PJS to some extent.

CONCLUSION

In conclusion, we discovered a series of new gene mutation sites, analyzed their pathogenicity, and enriched the mutation spectrum of PJS pathogenic genes. And through the summary of the clinical phenotypes with different *STK11* genotypes, to explore whether they are related, and get some tendentious research results. The detection of *SLX4* gene mutations in patients with PJS was reported for the first time. The relationship between *SLX4* gene mutations and the occurrence of PJS is still unclear, but may help to explain the genetic heterogeneity of PJS.

ARTICLE HIGHLIGHTS

Research background

Different types of pathogenic mutations may produce different clinical phenotypes, but no exact correlation between Peutz-Jeghers syndrome (PJS) genotype and clinical phenotype has been found so far. So it is necessary to study the correlation between genotype and clinical phenotype of PJS, and explore the internal molecular mechanism of different clinical phenotypes.

Research motivation

The authors included 24 cases of treated PJS cases as study participants, collected peripheral venous blood or normal tissue adjacent to polyps for high-throughput next-generation sequencing (NGS) of 139 hereditary colorectal tumor-related genes including *STK11/LKB1* to study the correlation between genotype and clinical phenotype of PJS.

Research objectives

To investigate the correlation between the genotype and clinical phenotype of PJS.

Research methods

Twenty-four patients with PJS were randomly selected for study inclusion. A total of 139 common hereditary tumor-related genes including *STK11/LKB1* were screened and analyzed for pathogenic germline mutations by high-throughput next-generation sequencing (NGS), and the pathogenicity of these mutations was evaluated.

Research results

STK11/LKB1 gene mutations were identified in 20 PJS patients, 90% of which were pathogenic mutations. 10 cases had new mutation sites. Pathogenic mutations were significantly less frequent in exon 7 of the *STK11/LKB1* gene than in other exons. Truncation mutations were more common in exons 1 and 4, and their pathogenicity was significantly higher than that of missense mutations. We also identified *SLX4* gene mutations in PJS patients.

Research conclusions

PJS has a relatively complicated genetic background. Changes in the sites responsible for coding functional proteins in exon 1 and exon 4 of *STK11/LKB1* may be one of the main causes of PJS. Mutation of the *SLX4* gene may help to explain the genetic heterogeneity of PJS.

Research perspectives

Exploration of the relationships of clinical phenotypes with different *STK11* genotypes, may help to interpret some controversial research results. The detection of *SLX4* gene mutations in patients with PJS was reported for the first time.

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ORIGINAL ARTICLE

Clinical and Translational Research

Validation of the Italian translation of the perceived stigma scale and resilience assessment in inflammatory bowel disease patients

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Abstract

BACKGROUND

Stigmatization is the separation of an individual from a group due to aspects that make them different. Resilience may in turn influence the perception of stigma. Patients with inflammatory bowel disease (IBD) are susceptible to stigma, although data are very limited.

AIM

To validate an Italian translation of the IBD perceived stigma scale (PSS) in relation to patients' resilience.

METHODS

Consecutive IBD outpatients were prospectively enrolled (December 2018-September 2019) in an Italian, tertiary referral, IBD center. Clinical and demographic data were collected. Stigma and resilience were evaluated through the IBD-PSS and the 25-item Connor-Davidson Resilience Scale, respectively. The International Quality of Life Assessment Project approach was followed to translate the IBD-PSS into Italian and to establish data quality. Higher scores represent greater perceived stigma and resilience. Multivariable analysis for factors associated with greater stigma was computed.

RESULTS

statement: The study was approved by the local Ethics Committee (Protocol Number 20190003611). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Informed consent statement: All participants gave their informed written consent to take part to the study and for the anonymized publication of data.

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Overall, 126 IBD patients (mean age 46.1 ± 16.9) were enrolled. The International Quality of Life Assessment criteria for acceptable psychometric properties of the scale were satisfied, with optimal data completeness. There was no ceiling effect, whilst floor effect was present (7.1%). The discriminant validity and the internal consistency reliability were good (Cronbach alpha = 0.87). The overall internal consistency was 95%, and the test-retest reliability was excellent 0.996. The median PSS score was 0.45 (0.20-0.85). Resilience negatively correlated with perceived stigma (Spearman's correlation = -0.18, 95% confidence intervals: -0.42-0.08, P = 0.03).

CONCLUSION
We herein validated the Italian translation of the PSS scale, also demonstrating that resilience negatively impacts perceived stigma.

Key Words: Crohn's disease; Quality of life; Stress; Ulcerative colitis

Core Tip: We have here validated an Italian version of the Perceived Stigma Scale for patients with inflammatory bowel disease. We have also found that resilience levels negatively correlated with perceived stigma. This is the first study assessing this issue in patients with inflammatory bowel disease.

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INTRODUCTION

Stigmatization is defined as the societal identification of an individual as abnormal and worthy of separation from the group, leading to discrimination and loss of their social status[1]. It has been reported that inflammatory bowel disease (IBD) is susceptible to stigmatization, not only because of the *taboo* around its symptoms, but also due to the assumption of being a psychosomatic condition affecting people because of their "obsessive behavior"[2] and because it affects sexual life[3]. Stigmatization in IBD patients was reported to be as high as 84%, regardless of disease activity [4].

An important aim of taking care of chronic patients should be the improvement of their quality of life (QoL), taking into account the social context and their needs[5]. Nonetheless, it emerged from a recent review that the burden of stigmatization in IBD, and the ability to positively cope with the disease (*i.e.* resilience), are not adequately addressed by clinicians[6]. In IBD patients, resilience has been found to be influenced by individual characteristics, including age, sex, and employment status and to influence positively the disease prognosis[7-9]. Stigma can be evaluated through the use of different scales, including the IBD perceived stigma scale (PSS)[10], which has been adapted and used in IBD patients[11]. Similarly, resilience can be measured through the Connor-Davidson resilience scale (CD-RISC), a 25-item self-administrated scale exploring different aspects related to the individual ability to cope with adversity and stress[12]. The CD-RISC was initially designed for psychiatric American patients, and it has now been translated into more than 70 languages, being the most widely used resilience scale in a variety of conditions[13].

The first study looking into perceived stigma in IBD showed that functional impairment was mainly due to IBD patients' psychological dimension rather than to their physical one. They also reported that patients with Crohn's disease (CD) had a higher degree of perceived stigma than patients with ulcerative colitis (UC)[14]. While perceived stigma is difficult to address and modify, resilience is responsive to

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behavioral intervention and is independently associated with better QoL and lower disease activity in IBD[9].

There are very limited data regarding perceived stigma in IBD, and no validated translation of the PSS into Italian is available. As a consequence, perceived stigma in Italian IBD patients has never been assessed. Therefore, we aimed to validate the Italian version of the PSS in IBD patients in order to obtain a meaningful instrument for assessing stigmatization and compare with international studies. We also assessed resilience and its relation with stigmatization.

MATERIALS AND METHODS

Study population

All IBD patients followed-up at the IBD Clinical & Research Centre of the San Matteo Hospital Foundation were consecutively enrolled between December 2018 and September 2019. IBD diagnosis was established according to internationally agreed criteria[15]. Patients were eligible for inclusion if they had at least a 3-mo history of IBD, were aged ≥ 18, were able to complete a questionnaire, and were willing to give written informed consent. Patients with an inconclusive or uncertain diagnosis of IBD or those diagnosed less than 3 mo before or unwilling to provide informed consent were excluded. Demographic and clinical characteristics were gathered, including IBD type, disease activity and duration, comorbidities, and previous IBD-related surgery. Clinical activity was assessed using the Harvey-Bradshaw index (HBI)[16,17] for CD and the partial Mayo score (pMayo)[18] for UC. For CD patients, HBI < 5 was defined as clinical remission, HBI 5-7 as mild disease, HBI 8-16 as moderate disease, and HBI > 16 as severe disease[16,17]. For UC patients, pMayo < 2 was defined as remission, pMayo 2-4 was defined as mild activity, pMayo 5-7 was defined as moderate activity, and pMayo > 7 was defined as severe activity[18]. The study was approved by the local Ethics Committee (Protocol Number 20190003611), and all participants gave their informed written consent to take part to the study and for the anonymized publication of data. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Assessment of the PSS

The PSS was initially designed to assess stigma in irritable bowel syndrome (IBS) patients and was validated in IBD in 2009 in a cohort of patients from the United States [4]. The PSS is a self-administered questionnaire designed to measure perceived stigma through 10 items on a five-point Likert scale (ranging from 0 = never to 4 = always), with a higher score reflecting a greater level of perceived stigma. Each item is assessed on two different domains: Significant others (SO) and healthcare professionals (HP), leading to a total of 20 items. The two-domain PSS version has already been used for different gastrointestinal disease and was found to have an excellent internal consistency and split-half reliability (≥ 0.89)[19].

The score indicating the perceived stigma ranges from 0 to 4 and is obtained by calculating the mean of all the values and the values within each domain.

Translation and cultural validation

We aimed to create a version that was easy to understand and complete by Italian IBD patients, without losing the original English version's equivalence and psychometric validity. The translation and adaptation were made in accordance with the International QoL Assessment (IQOLA) Project approach, which consists of three steps: A forward translation, a backward translation, and a cognitive testing[20,21].

Step 1—Forward translation: Two bilingual physicians (Lenti MV and Cococcia S) blindly translated the questionnaire from English into Italian. The two versions were compared, and discrepancies reconciled. Difficulty and degree of agreement of the translation were rated on a 1 to 100 scale (lowest–highest). For each item, the agreed forward translation was accepted if the scores were ≥ 75, otherwise retranslation was independently performed and scoring repeated.

Step 2—Backward translation: The Italian translation was blindly translated back into British English by two mother-tongue English people with a high educational level (graduated). The same reconciliation process reported for the forward translation was applied. The equivalence of the agreed backward translation to the original version

was rated and expected to be ≥ 75. If that threshold was not reached, the four translators had to agree on a new forward translation.

Step 3—Cognitive testing: Cognitive testing of the agreed Italian version was performed on 10 individuals with different age, sex, and educational background to verify that the translation was clear and understandable by a range of different people. Finally, a panel discussion was held to approve the final version (see Supplementary data).

Resilience assessment

Resilience was assessed through the Italian validated translation of the CD-RISC scale, a self-administered questionnaire assessing resilience through 25 items on a five-point Likert scale (ranging from 0 = totally disagree to 4 = totally agree)[12]. The score is calculated by summing the score of each item (ranging from 0 to 100) with higher scores meaning higher resilience.

Statistical analysis and psychometric evaluation

The sample size was computed based on the primary endpoint. A sample of 100 subjects responding to 20 items would achieve 80% power to detect the difference between the coefficient alpha under the null hypothesis of 0.70 and the coefficient alpha under the alternative hypothesis of 0.81, using a two-sided *F*-test with a significance level of 0.05. Twenty-six extra patients were enrolled to account for possible dropouts.

The PSS scoring was performed according to the scoring manuals, meaning that higher scores represent a higher level of perceived stigma. The psychometric evaluation of the Italian version of the PSS questionnaire included evaluation of data quality, including completeness (Table 1). Results were described as mean and standard deviation, and ceiling and floor effect were evaluated. Reliability was assessed and expressed by means of Cronbach's alpha for internal consistency, alpha = k × r/[1+(k-1) × r]; with k = number of items and r = mean correlation. Item internal consistency (correlation of item and corresponding scale, corrected for overlap), equality of item-scale correlations, and item discriminant validity (correlation of item with the corresponding scale *vs* correlation of item with other scales) were evaluated through the multi-trait/multi-item correlation matrix. Means of Pearson's correlation coefficient and intraclass correlation coefficient were used to evaluate the test-retest correlation for temporal stability (within 1 mo), with 95% confidence intervals (95%CI). External validity was assessed through the comparison of PSS scores in patients with different characteristics by means of the Kruskall Wallis test, the test for trend; the correlation with continuous variables was assessed with the Spearman R. Stata 16 (StataCorp, College Station, TX, United States) was used for all computations. All tests were two-sided, and a P value < 0.05 was considered statistically significant. In the presence of missing data, missing items were replaced by the median of the corresponding scale, unless more than 50% of the items were missing, in which instance the questionnaire was dropped.

RESULTS

Demographic and clinical characteristics

Overall, 146 IBD patients were screened for inclusion in the study. Of these, 20 patients did not participate because denied consent (15 patients) or because were due to be followed up in another hospital. Hence, 126 IBD patients (mean age 46.1 ± 16.9, male 56.4%), 57 with CD and 69 with UC, were consecutively enrolled in the study. The demographic and clinical characteristics of the enrolled patients are reported in Table 2. CD patients were significantly younger than UC patients (42.3 ± 15.7 *vs* 49.3 ± 17.4; P = 0.03). Psychiatric disorders, including anxiety and depression, were the most common concomitant diseases (25.4%), followed by hypertension (22.2%) and cardiomyopathy (11.9%), which was significantly more common among UC patients (17.4% *vs* 5.26%; P = 0.05). Overall, the median disease duration was 8 years [interquartile range (IQR) 3-16]. The majority of the CD patients had a disease with an inflammatory behavior (56.1%), 43.9% had a structuring behavior, and 28.1% had a penetrating disease. Almost half of the CD patients (49.1%) had ileo-colonic involvement, and 33.3% had perianal disease. Among UC patients, half (52.2%) had an extensive disease, 37.7% had a left UC, 10.1% had an ulcerative proctitis, and 4.4% had

Table 1 Thresholds defining acceptable psychometric properties according to the International Quality of Life Assessment project

	Definition	Threshold
Data quality		
Missing items	Unanswered items	< 5%-10%
	Incomplete scales (< 50% of items answered)	< 5%-10%
Floor and ceiling effect	Extreme scores (either on the lower- or higher-end)	< 10%
Scaling assumption		
Internal consistency		
Item	Correlation among items of the same scale (Pearson correlation ≥ 0.4)	> 90%
Reliability	Overall consistency of the scale (Cronbach's alpha coefficient)	> 0.7
Discriminant validity	Items whose Pearson correlation with other scales is higher than with their scale	0%
Test-retest evaluation	Correlation between the results scales filled in twice by the same patients at defined time points (Pearson correlation)	> 0.7

a pouch. The proportion of patients with severe disease activity was higher among UC patients (10.1% *vs* 0%), while two-thirds were in remission in both groups. A quarter of the enrolled patients had an extraintestinal manifestation (28.6%), including anemia, arthritis, uveitis, and dermatological manifestations. When available, calprotectin and C-reactive protein (CRP) were used as inflammatory markers. Overall, 34.1% of the patients had a calprotectin < 50 mg/kg, 16.2% between 51-250 mg/kg, and 12.7% > 250 mg/kg with no difference according to the disease (P = 0.54). Similarly, 61.1% of the patients had a normal CRP, whilst roughly a third had raised levels of CRP (31.0%), with no difference between UC and CD patients (P = 1.00).

Translation and cultural validation

The PSS was translated according to the IQOLA project guidelines[20,21]. For the forward translation, the median difficulty was rated as 10 (range: 10-60) and the agreement was found to be 95 (range: 70-100). Items 1, 5, and 10 were adjusted after discussion. The backward translation equivalence was rated at 95 (range: 80-100). Minor changes were made to item 2 and 4 of the Italian translation to improve the original version's equivalence. A cognitive testing of the agreed Italian version was performed on 10 individuals with different ages (median 48-years-old, range: 29-88), sex (5 female), and educational background (5 graduated), which did not lead to any adjustment of the scale. Supplementary data show the validated Italian version of the PSS-IBD, while the questions of the original English version have already been published elsewhere[4].

Psychometric evaluation

The majority of the IQOLA criteria for acceptable psychometric properties of the scale were satisfied in our cohort as reported in Table 3. We reached an optimal data completeness, and we did not have any ceiling effect, whilst a floor effect was present in 7.1% of the cases (overall domain). The floor effect was greater for the HP domain when compared to the SO domain (42.1% *vs* 8.7%). When looking at scaling assumption, the internal consistency reliability of the Italian version of the PSS was good, with an overall Cronbach alpha coefficient of 0.87 (0.83 for SO and 0.81 for HP). Although an excellent item, internal consistency was found in each domain, with a Pearson correlation ranging from 0.4 to 0.6, and one item (item 8, SO domain) did not reach the predetermined threshold of 0.4, determining an overall item internal consistency of 95% (still indicative of an excellent item internal consistency). The discriminant validity of the scale was good for items 1 to 7 in both domains, whereas items 8 to 10 had exactly the same Pearson correlation with their domain and the other one for both SO and HP.

The test-retest reliability was excellent, being 0.999 (0.997-1.000) overall, 0.99 (0.997-1.000) in the SO domain and 0.994 (0.979-0.998) in the HP domain. The median PSS score was 0.45 (0.20-0.85) with a significantly higher score for the SO domain (0.70 IQR 0.40-1.40 *vs* 0.10 IQR 0.00-0.40, P < 0.001), whilst the median resilience score was 64 (IQR 53-78). The level of perceived stigma did not differ according to sex (P = 0.51), IBD type (P = 0.33), disease activity, age (P = 0.11), or disease duration (P = 0.49) (See

Table 2 Demographic and clinical characteristics of the validating cohort

	Overall (*n* = 126)	CD (*n* = 57)	UC (*n* = 69)	*P* value
Age (mean ± SD)	46.13 (± 16.95)	42.29 (± 15.7)	49.29 (± 17.38)	0.03
Male	71 (56.4%)	33 (57.9%)	38 (55.1%)	0.86
BMI	23.9 (± 4.1)	23.9 (± 4.3)	23.9 (± 3.9)	0.67
Disease duration (median, IQR)	8 (3-16)	10 (3-17)	8 (3.5-13)	0.44
Disease characteristics (CD)				
Location (CD)	-		-	
Terminal ileum (L1)		13 (22.8%)		
Colon (L2)		8 (14.0%)		
Ileo-colon (L3)		28 (49.1%)		
Upper GI (L4)		2 (3.5%)		
Perianal disease (p)		19 (33.3%)		
Behavior (CD)	-	-		
Inflammatory (B1)		32 (56.1%)		
Stricturing (B2)		25 (43.9%)		
Penetrating (B3)		16 (28.1%)		
Disease activity (HBI)	-	-		
< 5		38 (66.7%)		
5-7		14 (24.6%)		
8-16		5 (8.8%)		
> 16	-	0 (0%)		
Disease characteristics (UC)			-	
Location	-	-	7 (10.1%)	
Proctitis (E1)			26 (37.7%)	
Left sided (E2)			36 (52.2%)	
Extensive (E3)				
Disease activity (pMayo)	-			
< 2			45 (65.2%)	
2-4			13 (18.8%)	
5-7			4 (5.8%)	
> 7			7 (10.1%)	
Pouch			3 (4.4%)	
Extraintestinal manifestations	36 (28.6%)	17 (29.8%)	19 (27.5%)	0.84
Previous abdominal surgery	38 (30.2%)	22 (38.6%)	16 (42.1%)	0.07
Calprotectin				0.54
< 50	43(34.1%)	15 (26.3%)	28 (40.6%)	
51-250	33 (16.2%)	17 (29.8%)	16 (23.2.%)	
> 250	16 (12.7%)	7 (12.3%)	9 (13.4%)	
Missing	34 (27.0%)	18 (31.6%)	16 (23.2%)	
CRP				1.00
Normal	77 (61.1)	35 (62.4%)	42 (60.9%)	
Raised	39 (31.0%)	18 (31.6%)	21 (30.4%)	

Missing	10 (7.9%)	4 (7.0%)	6 (8.7%)	
Comorbidities	40 (31.8%)	16 (28.1%)	24 (34.8%)	0.45
Cardiomyopathy	15 (11.9%)	3 (5.26%)	12 (17.4%)	0.05
Hypertension	28 (22.2%)	12 (42.9%)	16 (23.2%)	0.83
Diabetes	11 (8.73%)	4 (7.0%)	7 (10.1%)	0.75
Hepatic failure	1 (0.8%)	1 (1.8%)	0 (0.0%)	0.45
Kidney failure	4 (3.17%)	3 (5.26%)	1 (1.45%)	0.32
Respiratory failure	2 (1.6%)	1 (1.8%)	1 (1.5%)	1.00
Neurologic diseases	5 (4.0%)	1 (1.8%)	4 (5.8%)	0.37
Psychiatric disorder	15 (11.9%)	7 (12.3%)	8 (11.6%)	1.00
Onco-hematological diseases	12 (9.5%)	2 (3.5%)	10 (14.5%)	0.06

BMI: Body mass index; CD: Crohn's disease; CRP: C-reactive protein; GI: Gastrointestinal; HBI: Harvey-Bradshaw Index; IQR: Interquartile range; PMS: Partial Mayo Score; SD: Standard deviation; UC: Ulcerative colitis.

Table 3 Psychometric characteristics of perceived stigma scale and its sub-scales

	Overall	Significant others	Healthcare professionals
Median score	0.45 (0.20-0.85)	0.70 (0.30-1.40)	0.10 (0-0.40)
Data quality			
Missing items	0 (0%)	0 (0%)	0 (0%)
Floor effect	9 (7.1%)	11 (8.7%)	53 (42.1%)
Ceiling effect	0 (0%)	0 (0%)	0 (0%)
Scaling assumption			
Internal consistency			
Item	19/20 (95.0%)	10/10 (100%)	10/10 (100%)
Reliability (Cronbach alpha)	0.87	0.83	0.81
Discriminant validity	-	30.0%	30.0%
Test-retest	0.99 (0.99-1.00)	0.999 (0.99-1.00)	0.99 (0.97-0.99)
Evaluation[a]			

[a]Intraclass correlation coefficient (95% confidence interval).

Table 4). On the contrary, disease activity was found to significantly reduce resilience (Spearman's correlation -0.18, 95%CI: -0.42-0.08, P = 0.03) in CD patients, whilst no significant difference was found in UC patients according to the disease activity (P = 0.23). When exploring the relations between perceived stigma and resilience, a significant negative Spearman's correlation was found (-0.20, 95%CI: -0.36 to -0.02; P = 0.03).

DISCUSSION

Stigmatization is an important, though often unattended, issue in clinical medicine. Whilst for other conditions (*e.g.*, human immunodeficiency virus, mental illness, and lung cancer) stigmatization has been widely studied[22-25], IBD data are scant and fragmentary. This might be partly due to the lack of a validated tool to be used for this purpose. We herein validated an Italian version of the PSS questionnaire that performs well, has good psychometric properties, and is easily understandable. The psychometric evaluation of the Italian PSS version showed an excellent Cronbach alpha coefficient, item internal consistency, and test-retest reliability. Our results are in line

Table 4 Correlation between inflammatory bowel disease perceived stigma scale scores and demographic or clinical characteristics

	PSS	*P*	Spearman's correlation (95%CI)	PSS SO	*P*	Spearman's correlation (95%CI)	PSS HP	*P*	Spearman's correlation (95%CI)
Median score (IQR)	0.45 (0.20 to 0.85)			0.70 (0.30 to 1.40)			0.10 (0.00 to 0.40)		
Age		0.11	-0.14 (-0.31 to 0.03)		0.08	-0.157 (-0.33 to 0.02)		0.20	-0.116 (-0.29 to 0.06)
Sex		0.51			0.26			0.29	
Female	0.45 (0.30 to 0.85)			0.70 (0.50 to 1.40)			0.10 (0.00 to 0.40)		
Male	0.45 (0.15 to 0.90)			0.70 (0.20 to 1.40)			0.10 (0.00 to 0.50)		
Diagnosis		0.33			0.35			0.34	
CD	0.55 (0.25 to 0.95)			0.80 (0.40 to 1.40)			0.10 (0.00 to 0.50)		
UC	0.45 (0.20 to 0.85)			0.70 (0.30 to 1.30)			0.10 (0.00 to 0.40)		
HBI		0.91	0.05 (-0.22 to 0.30)		0.91	0.05 (-0.21 to 0.31)		0.70	0.11 (-0.16 to 0.36)
< 5	0.53 (0.25 to 0.80)			0.75 (0.50 to 1.30)			0.10 (0.00 to 0.50)		
5-7	0.60 (0.10 to 1.15)			0.90 (0.20 to 1.60)			0.30 (0.00 to 0.50)		
8-16	0.40 (0.10 to 1.40)			0.70 (0.20 to 1.80)			0.10 (0.00 to 0.80)		
pMS		0.52	0.06 (-0.18 to 0.29)		0.44	0.03 (-0.21 to 0.26)		0.81	0.11 (-0.13 to 0.34)
< 2	0.40 (0.20 to 0.85)			0.60 (0.40 to 1.30)			0.10 (0.00 to 0.30)		
2-4	0.45 (0.05 to 0.75)			0.60 (0.10 to 1.20)			0.20 (0.00 to 0.40)		
5-7	0.75 (0.48 to 1.22)			1.40 (0.90 to 1.75)			0.15 (0.05 to 0.70)		
> 7	0.35 (0.15 to 1.50)			0.70 (0.00 to 2.70)			0.10 (0.00 to 0.70		
CD-RISC25	-	0.03	-0.20 (-0.36 to -0.02)	-	0.02	-0.20 (-0.36 to -0.03)	-	0.12	-0.14 (-0.31 to 0.04)

CD: Crohn's disease; CD-RISC25: 25-item Connor-Davidson Resilience Scale; CI: Confidence interval; HBI: Harvey-Bradshaw Index; HP: Healthcare professionals; IQR: Interquartile range; pMS: Partial Mayo Score; SO: Significant others; UC: Ulcerative colitis.

with previous literature validating stigma scales in different settings[4,10,26,27], showing that our translation is reliable and offers a tool to assess stigma in Italian IBD patients. In our cohort, the main concern is the high floor effect recorded, especially in the HP domain. However, this result was partially expected since all the included patients were followed-up at a tertiary IBD center. These patients likely experience lower levels of perceived stigma since they are looked after by IBD dedicated HP, whereas a different result might be obtained if the questionnaire would be administered in different settings, such as community centers or private practices. Even considering the setting bias, the level of perceived stigma was found to be lower than expected (median 0.45, IQR 0.20-0.85), when compared to previous literature showing a low-to-moderate level of perceived stigma among IBD patients, using the PSS[4]. This might be explained by the fact that the PSS has been originally designed to address perceived stigma in patients affected by a functional disorder rather than by an organic disease, such as IBD. In the PSS questionnaire, there are no questions addressing some of IBD patients' main concerns, including the fear of relapsing or of incontinence. Therefore, even if this scale offers a useful tool to assess stigma in IBD, it is our opinion that some adjustments are needed.

Additionally, levels of perceived stigma in IBD patients tend to decrease in long-standing disease, in contrast with what happens for IBS[11]. Since IBD is an organic disease not associated with unhealthy or socially unacceptable vices, it is therefore

more likely to be recognized and accepted as a "real" organic disease over time, especially when compared to IBS[11]. Most of the included patients have a longstanding history, which could have led to lower levels of perceived stigma in our sample.

Stigmatization is multifactorial and is influenced by both patient-dependent and environment-dependent factors[28-30]. Among these factors, we have previously speculated that a relation between stigma and resilience may exist[6]. In line with previous literature, we found a moderate level of resilience in our cohort[9]. We have here shown for the first time that, in IBD patients, higher levels of resilience correlate with lower levels of perceived stigma (overall and for SO) and, conceivably, to better QoL. Such correlation was not found for the HP domain, which might be due to the high floor effect reported in this domain. In case of adversity, resilience can modulate catecholamine and cortisol production reducing long-term effects on the body[31] and leading to better outcome also in IBD, which requires continuous adaptation to the unpredictable course of the disease. This hypothesis is supported by a recent study in which higher levels of resilience are associated with lower disease activity, although it is unclear if this result was due to reverse causation[9]. Our findings suggest that downstream public health intervention that focus on patients' resilience may reduce the level of perceived stigma and consequently may improve the patients' QoL. Follow-up data are being gathered to support this hypothesis, since resilience is easily influenced by other events and a single assessment might be misleading. In addition to intervention focused on building individual resilience, upstream public health interventions are needed to reduce stigma around IBD improving the awareness on the disease.

This study has some limitations. Firstly, the sample size was calculated to validate the Italian translation of the PSS and was not adequate to draw firm conclusions about the level of perceived stigma among IBD patients. Larger prospective studies are needed to explore this aspect. Secondly, the majority of the included patients had a low disease activity and that could represent a bias in interpreting the results. Stigma is internalized over time and, since IBD is a chronic disease, the level of perceived stigma is influenced mostly by the social environment rather than by acute events. On the contrary, resilience is strongly influenced by contextual events and can be improved through behavioral intervention, and this is why the ongoing follow-up of these patients will be useful to better assess the relation between stigma and resilience in IBD. Additionally, the level of resilience of the sampled patients ranged from average to good, and this might have played a role in lowering the stigma scores.

CONCLUSION

To conclude, we have herein developed a validated Italian version of the PSS. Also, we have assessed for the first-time stigmatization and its relation with resilience in a cohort of IBD patients. Interventions aimed at building a stronger resilience may reduce perceived stigma. The follow-up data on the variation of stigma and resilience levels over time are being collected.

ARTICLE HIGHLIGHTS

Research background

Patients living with inflammatory bowel disease (IBD) often experience a poor quality of life due to stigmatization that can be assessed through the IBD perceived stigma scale (PSS). Resilience is the ability to cope positively with a specific disease or situation.

Research motivation

Stigmatization in IBD patients, especially in relation to one's own resilience, has been poorly characterized. A validated Italian version of the IBD-PSS is not available.

Research objectives

To validate an Italian version of the PSS in IBD patients and to assess patients' resilience and its relation with stigmatization.

Research methods

We enrolled 126 consecutive IBD patients (mean age 46.1 ± 16.9, male 56.4%), 57 with CD and 69 with UC, in an Italian, tertiary referral, IBD center. Clinical and demographic data were collected, and stigma and resilience were evaluated through the IBD-PSS and the 25-item Connor-Davidson Resilience Scale, respectively. Psychometric validity of the IBD-PSS was assessed, and a multivariable analysis for factors associated with greater stigma was computed.

Research results

We found that the Italian version of the IBD-PSS had an acceptable reliability, having a Cronbach alpha of 0.87, with an excellent test-retest score. The median PSS score was 0.45 (0.20-0.85), and resilience negatively correlated with perceived stigma (Spearman's correlation -0.18, 95%CI: -0.42-0.08, $P = 0.03$).

Research conclusions

We have developed a reliable tool to be used in clinical practice for assessing stigmatization in Italian IBD patients. Also, we found that resilience may have an influence on stigmatization, possibly improving patients' illness perception.

Research perspectives

The Italian IBD-PSS should be used extensively in order to assess this important endpoint in the care of IBD patients. More prospective, long-term studies looking at more detailed factors influencing stigmatization and resilience are urgently needed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic factors of minimally invasive surgery for gastric cancer: Does robotic gastrectomy bring oncological benefit?

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Abstract

BACKGROUND

Gastric cancer is the third leading cause of cancer-related death worldwide and surgical resection remains the sole curative treatment for gastric cancer. Minimally invasive gastrectomy including laparoscopic and robotic approaches has been increasingly used in a few decades. Thus far, only a few reports have investigated the oncological outcomes following minimally invasive gastrectomy.

AIM

To determine the 5-year survival following minimally invasive gastrectomy for gastric cancer and identify prognostic predictors.

METHODS

This retrospective cohort study identified 939 patients who underwent gastrectomy for gastric cancer during the study period. After excluding 125 patients with non-curative surgery (n = 77), other synchronous cancer (n = 2), remnant gastric cancer (n = 25), insufficient physical function (n = 13), and open gastrectomy (n = 8), a total of 814 consecutive patients with primary gastric cancer who underwent minimally invasive R0 gastrectomy at our institution between 2009 and 2014 were retrospectively examined. Accordingly, 5-year overall and recurrence-free survival were analyzed using the Kaplan–Meier method with the log-rank test and Cox regression analyses, while factors associated with survival were determined using multivariate analysis.

RESULTS

Our analysis showed that age > 65 years, American Society of Anesthesiologists

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(ASA) physical status 3, total or proximal gastrectomy, and pathological T4 and N positive status were independent predictors of both 5-year overall and recurrence-free survival. Accordingly, the included patients had a 5-year overall and recurrence-free survival of 80.3% and 78.2%, respectively. Among the 814 patients, 157 (19.3%) underwent robotic gastrectomy, while 308 (37.2%) were diagnosed with pathological stage II or III disease. Notably, our findings showed that robotic gastrectomy was an independent positive predictor for recurrence-free survival in patients with pathological stage II/III [hazard ratio: 0.56 (0.33-0.96), P = 0.035]. Comparison of recurrence-free survival between the robotic and laparoscopic approach using propensity score matching analysis verified that the robotic group had less morbidity (P = 0.005).

CONCLUSION

Age, ASA status, gastrectomy type, and pathological T and N status were prognostic factors of minimally invasive gastrectomy, with the robot approach possibly improving long-term outcomes of advanced gastric cancer.

Key Words: Laparoscopy; Gastric cancer; Minimally invasive surgery; Prognostic factor; Stomach neoplasms

Core Tip: This retrospective cohort study on 814 patients undergoing minimally invasive surgery for primary gastric cancer revealed a 5-year overall and recurrence-free survival of 80.3% and 78.2%, respectively. Moreover, our analysis identified age, American Society of Anesthesiologists status, type of gastrectomy, and pathological T and N status as prognostic predictors for overall and recurrence-free survival. The robotic approach was also identified as an independent positive predictor for recurrence-free survival in patients with pathological stage II/III disease, confirmed by the lesser morbidity in the robotic group following propensity score analysis.

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INTRODUCTION

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer-related death worldwide[1]. Surgical resection remains the sole curative treatment for gastric cancer, with regional lymphadenectomy being recommended as a component of radical gastrectomy[2]. Laparoscopic gastrectomy has been increasingly used, considering its better short-term effects and comparable long-term outcomes compared to open gastrectomy[2].

The da Vinci surgical system (DVSS; Intuitive Surgical, Sunnyvale, CA, United States) had been developed to overcome several disadvantages identified for standard minimally invasive laparoscopic surgery[2]. Most laparoscopic surgeons expect that utilizing the DVSS for gastric surgery would allow them to overcome the technical difficulties of laparoscopic gastrectomy, thereby improving its safety, reproducibility, teachability, and long-term outcomes. However, only one large, nonrandomized prospective study (NCT01309256) has compared DVSS with laparoscopic gastrectomy. Accordingly, the study results mentioned above demonstrated that DVSS had higher operative time and cost than laparoscopic gastrectomy with no difference in morbidity, suggesting that DVSS might reduce cost-effectiveness[3]. Concurrently, robotic gastrectomy, which has been actively used for operable patients with resectable gastric cancer at the patient's own expense[2], was introduced at our institution in 2009. Analysis of patient outcomes following robotic gastrectomy had demonstrated that its morbidity was approximately one-fifth of that observed with

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laparoscopic gastrectomy, with such a reduction in morbidity, including decreased incidences of postoperative pancreatic fistula, certainly improving the short-term postoperative course[2]. Moreover, our previous study had compared the oncological outcomes, particularly 3-year survival rates, between robotic gastrectomy and laparoscopic gastrectomy[2]. Thus far, only a few reports have investigated the oncological outcomes following robotic gastrectomy, considering that DVSS remains a relatively new technology. Therefore, the current study aimed to determine the prognostic factors of minimally invasive gastrectomy, including laparoscopic and robotic procedures.

MATERIALS AND METHODS

Patients

This single-center retrospective cohort study included patients who underwent curative gastrectomy for gastric cancer at our institution between January 2009 and September 2014. The inclusion criteria were patients with primary gastric adenocarcinoma who underwent curative resection using minimally invasive surgery (MIS). The exclusion criteria were patients with other synchronous cancer and those whose resection was limited due to poor physical functioning. Among the 939 patients who underwent gastrectomy for gastric cancer during the study period, 125 were excluded due to non-curative surgery (n = 77), other synchronous cancer (n = 2), remnant gastric cancer (n = 25), insufficient physical function (n = 13), and open gastrectomy (n = 8). Thus, the 814 patients who satisfied the study criteria were ultimately analyzed. The clinicopathological variables collected included age, sex, body mass index (BMI), American Society of Anesthesiologists (ASA) physical status classification, date of surgery, type of approach, histologic type, lymphovascular invasion status, TNM staging (Japanese Gastric Cancer Association classification, 14th edition), number of harvested lymph nodes, postoperative complications determined by Clavien–Dindo (C-D) classification[4], date of the first recurrence, and date and status of the last follow-up. The extent of gastrectomy and lymphadenectomy was defined based on Japanese gastric cancer treatment guidelines[5]. Overall survival (OS) was calculated from the date of resection to the date of the last follow-up or death of any cause. Recurrence-free survival (RFS) was calculated from the date of resection to the date of first recurrence, last follow-up, or death of any cause, whichever occurred first. Details regarding indications for radical gastrectomy, including the selection of laparoscopic or robotic approach, surgical procedures, perioperative management, adjuvant chemotherapy, and oncologic follow-up, have been previously reported[2]. Neoadjuvant chemotherapy (NAC) (S-1 80 mg/m^2 days 1-21 + CDDP 60 mg/m^2 day 8 or S-1 80 mg/m^2 days 1-28) was offered to patients with clinical T ≥ 2, tumor size ≥ 5 cm, and/or swollen locoregional lymph nodes ≥ 1.5 cm[2]. All patients were uniformly offered robotic surgery without considering their backgrounds, including physical and oncological status. Patients who agreed to the uninsured use of the surgical robot underwent robotic gastrectomy, whereas those who wished for insured treatment underwent laparoscopic gastrectomy[2]. All patients were completely involved in the decision-making process and provided informed consent prior to participation. All surgical procedures were performed or guided by surgeons qualified by the Japanese Society for Endoscopic Surgical Skill Qualification System, initiated in 2004 by the Japanese Society for Endoscopic Surgery to develop a tool for the reliable and reproducible evaluation of trainees' surgical techniques[6]. All procedures were supervised by an expert gastric surgeon (I.U.) who had performed more than 1500 Laparoscopic gastrectomies and 400 robotic gastrectomies. This study was approved by the institutional review board of Fujita Health University.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics 25 (IBM Corporation, Armonk, NY, United States). Long-term outcomes were analyzed using the Kaplan–Meier method with the log-rank test and Cox regression analyses. Considering our relatively small sample size, multivariate analysis was conducted using all variables determined to be significant (P < 0.1) during univariate analysis as independent variables. Data were expressed as median, interquartile range, or hazard ratio (HR) with the 95% confidence interval (CI) unless otherwise stated. A P value of < 0.05 (two-tailed) was considered statistically significant. Propensity score matching analysis was used to reduce selection bias with regard to potential confounding factors when establishing the laparoscopic and robotic groups. Possible confounders were

selected based on their potential association with the outcome of interest according to clinical knowledge. Therefore, clinicopathological characteristics (age, BMI, sex, ASA status, pathological T and N factor, type of surgery, tumor size, and NAC) were used to adjust differences between the laparoscopic and robotic groups through one-to-one pair matching using optimal match without replacement. Propensity scores were matched using a caliper width 1/5 Logit of the standard deviation. The absolute standardized difference was used to measure covariate balance, in which an absolute standardized mean difference above represented a meaningful imbalance[7]. Independent continuous variables were compared using the Mann–Whitney *U* test or Kruskal-Wallis test. Categorical variables were compared using the χ^2 test or Fisher's exact test.

RESULTS

Patient characteristics

Table 1 summarizes the characteristics of all patients included herein. Accordingly, the included patients had a median age of 68 years, among whom 31.4% (n = 256) were diagnosed with clinical stage II or more disease, while 14.6% (n = 119) underwent NAC. Laparoscopic and robotic gastrectomy was performed in 657 (80.7%) and 157 (19.3%) patients, respectively. None of the patients required intraoperative conversion to open procedure from MIS. Pathological stage II and III disease was diagnosed in 160 (19.7%) and 148 (18.2%) patients, respectively. Morbidity of C-D grade ≥ III was observed in 72 patients (8.8%).

Survival outcomes

The median follow-up period was 59.5 mo, while the 5-year OS and RFS were 80.3% and 78.2%, respectively (Figure 1A and B). Patients with pStage I, II, and III had a 5-year OS of 91.9%, 76.3%, and 43.7%, and a 5-year RFS of 91.6%, 74.7%, and 36.0%, respectively.

Factors related to survival

Univariate analysis identified age > 65 years, ASA status 3, total or proximal gastrectomy, D2 lymphadenectomy, tumor size > 30 mm, lymphovascular invasion, C–D grade ≥ III morbidity, NAC administration, adjuvant chemotherapy administration, and higher pT and pN status as factors significantly associated with OS (Table 2). However, multivariate analysis revealed that only age > 65 years [HR: 1.62 (1.09-2.40), P = 0.017], ASA status 3 [HR: 1.91 (1.18-3.10), P = 0.009], total or proximal gastrectomy [HR: 1.45 (1.03-2.05), P = 0.036], pT4 [HR: 4.31 (2.37–7.82), P < 0.001], and pN positive status were significantly and independently associated with OS (Table 2). Similarly, multivariate analysis identified age > 65 years [HR: 1.48 (1.02-2.14), P = 0.038], ASA status 3 [HR: 1.62 (1.02-2.60), P = 0.043], total or proximal gastrectomy [HR: 1.55 (1.12–2.15), P = 0.009], pT4 [HR: 4.20 (2.38–7.41), P < 0.001], and pN positive status as factors significantly and independently associated with RFS (Table 3). Moreover, multivariate analysis showed that robotic approach could likely be a positive predictor for RFS, although no significant association was observed [HR 0.68 (0.44-1.06), P = 0.088] (Table 3).

Survival outcomes following the laparoscopic and robotic approach

The laparoscopic and robotic approach had a 5-year OS of 79.4% and 83.4% (P = 0.243) and a 5-year RFS of 76.9% and 84.2% (P = 0.085), respectively. No significant difference in the 5-year OS and RFS was noted between both groups for patients with pStage I (91.6% *vs* 93.4%, P = 0.471 and 91.4% *vs* 92.7%, P = 0.634) (Figure 2A and B). Notably, among patients with pStage II/III, those in the robotic group had significantly better RFS compared to those in the laparoscopic group (74.1% *vs* 51.7%, P = 0.006) (Figure 2D), although no significant difference in the 5-year OS was observed (P = 0.071) (Figure 2C).

Factors associated with survival in pStage II/III diseases

Our analysis showed that pT4 [HR: 4.02 (1.21-13.42), P = 0.024] and pN positive status were significantly and independently associated with OS. Notably, univariate analysis showed that robotic gastrectomy (P = 0.007), total or proximal gastrectomy (P = 0.004), tumor size > 30 mm (P = 0.014), pT4 (P = 0.007), and pN positive status were significantly associated with RFS. Meanwhile, multivariate analysis found that robotic

Table 1 Clinicopathological characteristics of the entire cohort

Variables	*n* = 814
Age, yr [IQR]	68[61-74]
Sex, *n* (%)	
Male	562 (69.0)
Female	252 (31.0)
BMI, kg/m^2 [IQR]	22.2 [20.0-24.1]
ASA, *n* (%)	
1	314 (38.6)
2	396 (48.6)
3	104 (12.8)
Clinical stage, *n* (%)	
I	558 (68.6)
II	125 (15.3)
III	121 (14.9)
IV	10 (1.2)
Neoadjuvant chemotherapy, *n* (%)	119 (14.6)
Neoadjuvant radiotherapy, *n* (%)	0 (0)
Approach, *n* (%)	
Laparoscopic	657 (80.7)
Robotic	157 (19.3)
Type of gastrectomy, *n* (%)	
Distal	559 (68.7)
Total	238 (29.2)
Proximal	16 (2.0)
Pylorus preserving	1 (0.1)
Lymphadenectomy, *n* (%)	
D1+	378 (46.4)
D2	436 (53.6)
Dissected nodes, *n* [IQR]	38[28-48]
Tumor size, mm [IQR]	30[20-50]
pT, *n* (%)	
1	469 (57.6)
2	87 (10.7)
3	112 (13.8)
4	138 (17.0)
CR	8 (1.0)
pN, *n* (%)	
0	559 (68.7)
1	98 (12.0)
2	79 (9.7)
3	78 (9.6)
pStage, *n* (%)	

I	498 (61.2)
II	160 (19.7)
III	148 (18.2)
TCRNany	8 (1.0)
WHO histologic type, *n* (%)	
Tub/pap	402 (49.4)
Por/sig	352 (43.2)
Mixed/other	60 (7.4)
Lymphovascular invasion, *n* (%)	531 (65.2)
Adjuvant chemotherapy, *n* (%)	242 (29.7)
Adjuvant radiotherapy, *n* (%)	0 (0)
Morbidity (C–D grade ≥ III), *n* (%)	72 (8.8)
Anastomotic leakage	22 (2.7)
Pancreatic fistula	30 (3.7)

Categorical and continuous data are presented as *n* (%) and median [IQR], respectively. IQR: Interquartile range; ASA: American Society of Anesthesiologists; BMI: Body mass index; CR: Complete response at the primary site; C-D: Clavien-Dindo classification.

gastrectomy was independently and positively associated with RFS [HR: 0.56 (0.33–0.96), P = 0.035]. Apart from robotic gastrectomy, only pT4 and pN positive status were identified as factors independently associated with RFS (Table 4).

Comparison between robotic and laparoscopic gastrectomy in pStage II/III diseases

To account for confounding factors between both groups, propensity score matching was performed (Table 5). In the prematched cohort of 308 patients with pStage II/III disease, 67 and 241 patients belonged to the robotic and laparoscopic groups, respectively. After matching, each group comprised 61 patients. The matched cohort had a considerably better balance of covariates, with < 0.245 of the cutoff value of an absolute standardized difference. In the postmatched cohort, no differences in clinicopathological variables were observed between the laparoscopic and robotic groups, although the robotic group had lower morbidity (4.9% *vs* 16.4%, P = 0.04) (Table 5). Furthermore, the robotic group had significantly better 5-year OS (70.4% *vs* 50.2%, P = 0.039) and RFS (74.1% *vs* 44.5%, P = 0.005) than the laparoscopic group in the postmatched cohort (Figure 3A and B).

DISCUSSION

The current study clearly identified factors related to survival in patients with gastric cancer who underwent MIS, subsequently presenting three significant findings.

First, the present study highlighted the feasibility and safety of MIS for gastric cancer as determined by the 5-year outcomes. While the long-term outcomes of laparoscopic surgery have been increasingly reported in recent years, only a few studies have investigated the long-term outcomes of the robotic approach[2,8,9]. Consistent with previous studies, including those from our group, the current study demonstrated no significant difference in OS and RFS between the laparoscopic and robotic approaches [2,8,9]. However, among patients with pStage II/III, those in the robotic group demonstrated significantly better RFS than those in the laparoscopic group (P = 0.006).

Second, our results showed that pT and pN status was independently associated with both OS and RFS. Currently, multidisciplinary treatment for gastric cancer utilizing various chemotherapeutic options has been developed worldwide. In Western countries, neoadjuvant and adjuvant chemotherapy combined with curative resection has been the standard treatment for advanced gastric cancer[10,11], whereas adjuvant chemotherapy following curative resection remains the standard approach in Japan[5]. Regardless of treatment options, however, evidence has shown that the pN factor is consistently strongly associated with survival following gastric cancer treatment[12-14]. The results of the current study are consistent with those presented

Table 2 Factors associated with overall survival for the entire cohort (*n* = 814)

	Univariate			Multivariate		
	HR	95%CI	*P*	HR	95%CI	*P*
Age > 65 yr	1.46	1.04–2.06	0.031	1.62	1.09–2.40	0.017
Female sex	0.75	0.52–1.09	0.129			
BMI > 23 kg/m^2	0.77	0.55–1.07	0.123			
ASA						
1	1			1		
2	0.96	0.68–1.38	0.837	1.06	0.72–1.57	0.753
3	1.97	1.27–3.05	0.003	1.91	1.18–3.10	0.009
Neoadjuvant chemotherapy	1.84	1.27–2.67	0.001	1.34	0.88–2.04	0.166
Robotic approach	0.77	0.50–1.21	0.258			
Type of gastrectomy						
Distal/pylorus-preserving	1			1		
Total/proximal	2.17	1.58–2.99	< 0.001	1.45	1.03–2.05	0.036
D2 lymphadenectomy	1.86	1.32–2.61	< 0.001	0.87	0.57–1.33	0.528
Tumor > 30 mm	3.23	2.20–4.75	< 0.001	1.05	0.66–1.69	0.832
WHO histologic type						
Tub/pap	1			1		
Por/sig/mixed/other	1.54	1.12–2.13	0.009	1.26	0.89–1.78	0.190
Lymphovascular invasion	4.38	2.68–7.17	< 0.001	1.17	0.60–2.26	0.651
pT						
1	1			1		
2	2.82	1.62–4.91	< 0.001	1.72	0.90–3.27	0.099
3	3.03	1.82–5.03	< 0.001	1.54	0.82–2.91	0.184
4	9.78	6.54–14.60	< 0.001	4.31	2.37–7.82	< 0.001
CR	1.67	0.23–12.17	0.613	1.34	0.16–10.97	0.784
pN						
0	1			1		
1	2.76	1.74–4.39	< 0.001	2.02	1.22–3.34	0.007
2	4.05	2.56–6.41	< 0.001	1.97	1.15–3.36	0.013
3	8.08	5.40–12.10	< 0.001	2.92	1.79–4.78	< 0.001
Adjuvant chemotherapy	3.27	2.37–4.50	< 0.001	1.21	0.80–1.82	0.371
Morbidity (C-D grade ≥ III)	1.85	1.18–2.91	0.008	1.27	0.79–2.05	0.325

HR: Hazard ratio; CI: Confidence interval; ASA: American Society of Anesthesiologists; BMI: Body mass index; CR: Complete response at the primary site; C-D: Clavien–Dindo classification.

in previous studies.

Third, the use of the surgical robot was significantly associated with improved RFS among propensity score-matched patients with pStage II/III disease. This could have been attributed to lower morbidity in the robotic gastrectomy group, a causal relationship between morbidity and survival, and higher morbidity in patients undergoing surgery for advanced disease. First, a few studies have shown that robotic gastrectomy was technically safe and feasible but did not have superior morbidity compared to the laparoscopic approach[3]. However, Wang *et al*[15] who compared morbidity between robotic and laparoscopic gastrectomy using propensity score-

Table 3 Factors associated with recurrence-free survival for the entire cohort (*n* = 814)

	Univariate			Multivariate		
	HR	95%CI	*P*	HR	95%CI	*P*
Age > 65 yr	1.33	0.97–1.84	0.076	1.48	1.02–2.14	0.038
Female sex	0.76	0.54–1.06	0.108			
BMI > 23 kg/m^2	0.77	0.56–1.05	0.100			
ASA						
1	1			1		
2	0.95	0.68–1.32	0.761	1.08	0.75–1.55	0.692
3	1.67	1.09–2.55	0.018	1.62	1.02–2.60	0.043
Neoadjuvant chemotherapy	1.91	1.34–2.71	< 0.001	1.39	0.93–2.08	0.104
Robotic approach	0.69	0.45–1.06	0.087	0.68	0.44–1.06	0.088
Type of gastrectomy						
Distal/pylorus-preserving	1			1		
Total/proximal	2.24	1.66–3.02	< 0.001	1.55	1.12–2.15	0.009
D2 lymphadenectomy	2.08	1.50–2.86	< 0.001	0.99	0.66–1.48	0.957
Tumor > 30 mm	3.19	2.23–4.56	< 0.001	0.95	0.61–1.48	0.827
WHO histologic type						
Tub/pap	1			1		
Por/sig/mixed/other	0.54	0.14–2.08	0.005	1.20	0.86–1.66	0.284
Lymphovascular invasion	4.93	3.06–7.94	< 0.001	1.29	0.69–2.43	0.430
pT						
1	1			1		
2	2.87	1.69–4.85	< 0.001	1.57	0.85–2.89	0.148
3	3.42	2.13–5.48	< 0.001	1.60	0.89–2.89	0.120
4	10.62	7.26–15.53	< 0.001	4.20	2.38–7.41	< 0.001
CR	1.41	0.19–10.24	0.737	0.96	0.12–7.77	0.967
pN						
0	1			1		
1	3.08	1.99–4.77	< 0.001	2.23	1.39–3.58	0.001
2	5.01	3.29–7.62	< 0.001	2.24	1.36–3.70	0.002
3	8.92	6.07–13.11	< 0.001	3.32	2.06–5.34	< 0.001
Adjuvant chemotherapy	3.53	2.62–4.77	< 0.001	1.18	0.80–1.73	0.410
Morbidity (C-D grade ≥ III)	1.69	1.09–2.62	0.019	1.04	0.65–1.67	0.868

ASA: American Society of Anesthesiologists; BMI: Body mass index; CR: Complete response at the primary site; C-D: Clavien–Dindo classification; HR: Hazard ratio; CI: Confidence interval.

matched analysis, reported that the robotic group exhibited significantly lower morbidity, particularly with regard to infectious complications (*e.g.*, anastomotic leakage and intra-abdominal abscess)[15]. Furthermore, a multicenter, prospective, single-arm study by our group recently reported that robotic gastrectomy promoted lesser morbidity than laparoscopic gastrectomy among historical controls[2]. Similarly, the present study showed that the robotic group had significantly lesser morbidity compared to the laparoscopic in the postmatched cohort. Second, several studies have demonstrated that morbidity was associated with worse survival in gastric cancer[16-19]. In fact, Jin *et al*[16] reported that patients with and without postoperative complic-

Table 4 Factors associated with recurrence-free survival for patients with pathological stage II/III disease (*n* = 308)

	Univariate			Multivariate		
	HR	95%CI	*P*	HR	95%CI	*P*
Age > 65 yr	1.04	0.73–1.48	0.848			
Female sex	1.08	0.75–1.58	0.673			
BMI > 23 kg/m^2	0.69	0.47–1.00	0.052	0.92	0.62–1.35	0.657
ASA						
1	1					
2	0.82	0.56–1.19	0.297			
3	1.07	0.63–1.80	0.809			
Neoadjuvant chemotherapy	1.37	0.93–2.01	0.114			
Robotic approach	0.50	0.30–0.83	0.007	0.56	0.33–0.96	0.035
Type of gastrectomy						
Distal/pylorus-preserving	1			1		
Total/proximal	1.67	1.18–2.37	0.004	1.32	0.91–1.90	0.145
D2 lymphadenectomy	1.26	0.80–2.00	0.320			
Tumor > 30 mm	2.17	1.17–4.03	0.014	1.34	0.69–2.60	0.303
WHO histologic type						
Tub/pap	1			1		
Por/sig/mixed/other	1.38	0.95–2.00	0.089	1.29	0.88–1.90	0.197
pT						
1	1			1		
2	1.82	0.60–5.53	0.292	1.33	0.42–4.23	0.628
3	1.32	0.47–3.75	0.6	1.45	0.49–4.30	0.505
4	3.96	1.45–10.83	0.007	3.52	1.23–10.07	0.019
pN						
0	1			1		
1	2.07	1.16–3.69	0.014	2.86	1.57–5.24	0.001
2	2.24	1.29–3.90	0.004	2.45	1.38–4.34	0.002
3	3.74	2.21–6.32	< 0.001	3.25	1.88–5.61	< 0.001
Adjuvant chemotherapy	1.37	0.92–2.04	0.119			
Morbidity (C-D grade ≥ III)	1.58	0.97–2.58	0.066	1.22	0.73–2.05	0.453

ASA: American Society of Anesthesiologists; BMI: Body mass index; C–D: Clavien–Dindo classification; HR: Hazard ratio; CI: Confidence interval.

ations had RFS rates of 23% and 40%, respectively ($P < 0.001$). Third, advanced gastric cancer requires complicated procedures, which can cause more complications. Notably, studies have reported that patient with advanced disease had morbidity rates of 8.3%-15.2% following minimally invasive gastrectomy, respectively[20,21]. The aforementioned findings therefore indicate that utilizing surgical robots, which cause less morbidity, might at least partly contribute to the better RFS in patients with advanced gastric cancer, suggesting that surgical robots may be more beneficial for patients with advanced disease. However, although univariate analysis found morbidity to be significantly associated with RFS, multivariate analysis did not identify the same as a significant independent factor associated with RFS in the entire cohort. As such, further investigations are warranted to confirm such findings.

The current study has several limitations worth noting. First, this study was retrospective in nature and involved only a single institution. Moreover, the sample

Table 5 Clinicopathological characteristics of pStage II/III patients in the pre- and postmatched cohort

	Prematched				Postmatched			
	Lap (*n* = 241)	Robotic (*n* = 67)	*P*	ASD	Lap (*n* = 61)	Robotic (*n* = 61)	*P*	ASD
Sex, *n* (%)			0.132	0.204			0.580	0.100
Male	174 (72.2)	42 (62.7)			35 (57.4)	38 (62.3)		
Female	67 (27.8)	25 (37.3)			26 (42.6)	23 (37.7)		
Age, yr [IQR]	69 [61–75]	65 [60–77]	0.134	0.235	68 [61–75]	65 [60–77]	0.824	0.042
BMI, kg/m^2 [IQR]	21.6 [19.2–23.7]	23.1 [20.0–24.8]	0.008	0.329	22.6 [20.4–24.9]	23.0 [20.0–24.9]	0.810	0.007
ASA, *n* (%)			0.074	0.315			0.959	0.052
1	89 (36.9)	35 (52.2)			31 (50.8)	30 (49.2)		
2	118 (49.0)	24 (35.8)			23 (37.7)	23 (37.7)		
3	34 (14.1)	8 (11.9)			7 (11.5)	8 (13.1)		
Neoadjuvant chemotherapy, *n* (%)	61 (25.3)	11 (16.4)	0.128	0.220	10 (16.4)	11 (18.0)	0.810	0.043
Type of gastrectomy, *n* (%)			0.075	0.329			1	< 0.001
Distal	136 (56.4)	48 (71.6)			42 (68.9)	42 (68.9)		
Total	104 (43.2)	19 (28.4)			19 (31.1)	19 (31.1)		
Proximal	1 (0.4)	0 (0)			0 (0)	0 (0)		
Tumor size, mm [IQR]	50[35-70]	40[30-63]	0.026	0.265	50 [35–77]	43 [30–65]	0.192	0.187
pT, *n* (%)			0.042	0.391			0.860	0.158
1	11 (4.6)	8 (11.9)			4 (6.6)	6 (9.8)		
2	35 (14.5)	4 (6.0)			4 (6.6)	4 (6.6)		
3	85 (35.3)	27 (40.3)			21 (34.4)	23 (37.7)		
4	110 (45.6)	28 (41.8)			32 (52.5)	28 (45.9)		
pN, *n* (%)			0.15	0.338			0.617	0.244
0	65 (27.0)	24 (35.8)			16 (26.2)	22 (36.1)		
1	48 (19.9)	15 (22.4)			17 (27.9)	13 (21.3)		
2	68 (28.2)	10 (14.9)			9 (14.8)	10 (16.4)		
3	60 (24.9)	18 (26.9)			19 (31.1)	16 (26.2)		
pStage, *n* (%)			0.246				0.716	
II	121 (50.2)	39 (58.2)			32 (52.5)	34 (55.7)		
III	120 (49.8)	28 (41.8)			29 (47.5)	27 (44.3)		
Dissected nodes, *n* [IQR]	44 [35–53]	43 [35–51]	0.858		45 [35–54]	43 [30-65]	0.556	
WHO histological type, *n* (%)			0.667				0.229	
Tub/pap	88 (36.5)	27 (41.8)			17 (27.9)	26 (42.6)		
Por/sig	129 (53.5)	34 (50.7)			37 (60.7)	30 (49.2)		
Mixed/other	24 (10.0)	5 (7.5)			7 (11.5)	5 (8.2)		
Lymphovascular invasion, *n* (%)	241 (100)	66 (98.5)	0.218		61 (100)	60 (98.4)	0.5	
Adjuvant chemotherapy, *n* (%)	161 (66.8)	47 (70.1)	0.605		38 (62.3)	43 (70.5)	0.338	
Morbidity (C-D grade ≥ III), *n* (%)	31 (12.9)	3 (4.5)	0.053		10 (16.4)	3 (4.9)	0.04	

Categorical and continuous data are presented as *n* (%) and median [IQR], respectively. ASA: American Society of Anesthesiologists; BMI: Body mass

index; CR: Complete response at the primary site; C–D: Clavien–Dindo classification; ASD: Absolute standardized mean difference.

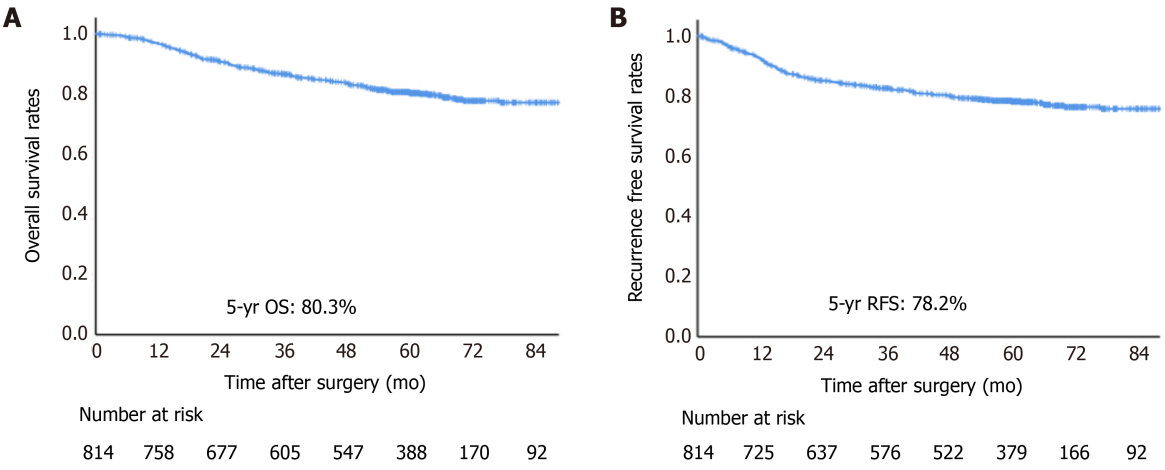

Figure 1 Kaplan-Meier curves. Kaplan-Meier estimates in the entire cohort A: Overall survival probability; B: Recurrence-free survival probability. OS: Overall survival; RFS: Recurrence-free survival.

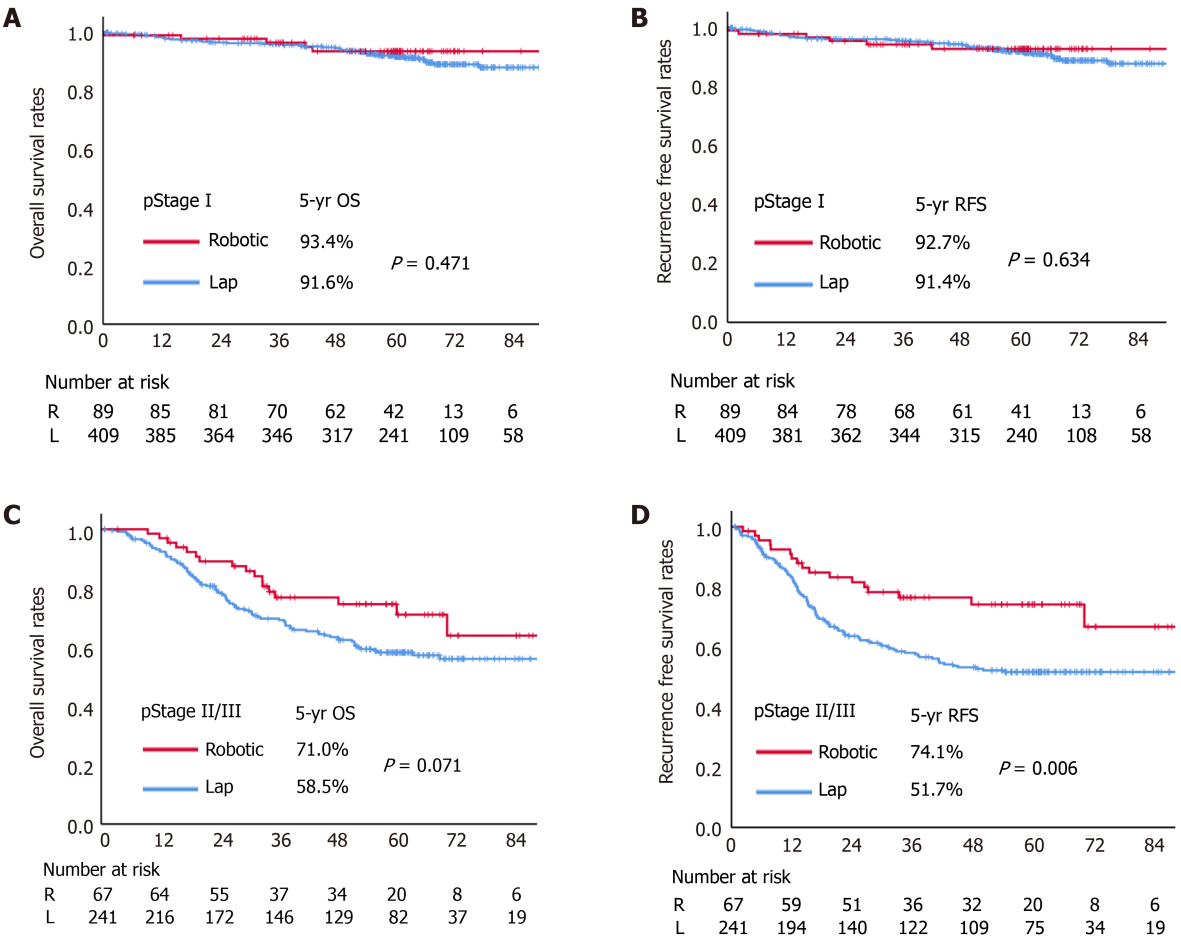

Figure 2 Kaplan-Meier curves. A and C: Kaplan–Meier estimates of overall survival probability for pathological stage I and II/III, B and D: Kaplan–Meier estimates of recurrence-free survival probability for pathological stage I and II/III. OS: Overall survival; RFS: Recurrence-free survival.

size, particularly that of the robotic group, was relatively small. Therefore, given that biases may exist in our data, the overall results should be interpreted with caution. As described in our previous reports[2,6], patients were selected according to whether the they agreed to the uninsured use of robot-assisted surgery, which may have caused

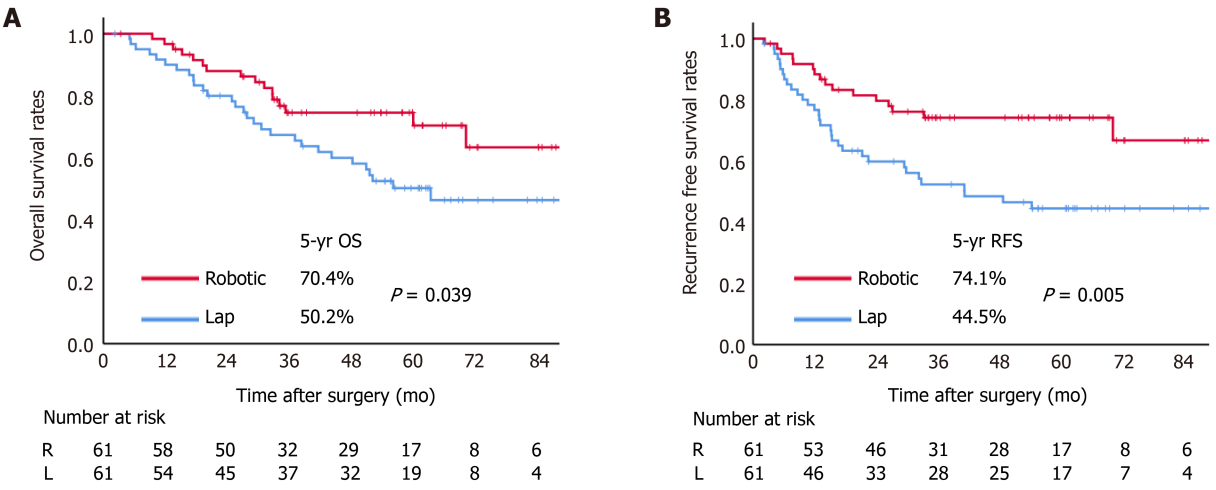

Figure 3 Kaplan-Meier curves. Kaplan-Meier estimates in the postmatched cohort. A: Overall survival probability; B: Recurrence-free survival probability. OS: Overall survival; RFS: Recurrence-free survival.

selection bias due to a possible preference for robotic gastrectomy in patients of higher economic status. However, this was an inherent limitation at the time of study enrollment considering that the DVSS was not covered by the medical insurance in Japan at the time the enrolled patients underwent gastrectomy, whereas conventional laparoscopic gastrectomy was covered. Second, propensity score matching between the laparoscopic and robotic group did not account for adjuvant chemotherapy administration given that, similarly to postoperative complications, adjuvant chemotherapy was determined after robotic or laparoscopic gastrectomy was conducted. Considering that both adjuvant chemotherapy and postoperative complications may affect prognosis[2,6,21], well-designed prospective trials are needed to determine a cause-effect relationship between robotic or laparoscopic gastrectomy and postoperative complications, as well as adjuvant chemotherapy administration.

CONCLUSION

In conclusion, the current study identified age, ASA status, type of gastrectomy, and pathological T and N status are prognostic factors of minimally invasive gastrectomy for gastric cancer. Moreover, the use of robotic assistance was associated with reduced early morbidity, as well as potentially better oncological outcomes in advanced gastric cancer.

ARTICLE HIGHLIGHTS

Research background

Minimally invasive surgery (MIS) including laparoscopic and robotic approaches for gastric cancer has been increasingly used because of its beneficial short-term effects over the open approach. However, oncological outcomes are not established.

Research motivation

There have been few reports on the oncological outcomes of MIS for gastric cancer patients, especially for the robotic approach, because a surgical robot remains a relatively new technology. Therefore, this study aimed to determine the prognostic factors of minimally invasive gastrectomy, including laparoscopic and robotic approaches.

Research objectives

This study aimed to determine the prognostic factors of minimally invasive gastrectomy, including laparoscopic and robotic approaches.

Research methods

This single-institutional retrospective cohort study included 814 consecutive patients with primary gastric cancer who underwent minimally invasive R0 gastrectomy between 2009 and 2014. We retrospectively examined 5-year overall survival and recurrence-free survival and investigated factors related to survival.

Research results

Age > 65 years, American Society of Anesthesiologists (ASA) physical status 3, total or proximal gastrectomy, and pathological T4 and N positive status were independent predictors of overall survival and recurrence-free survival. The five-year overall survival and recurrence-free survival were 80.3% and 78.2%, respectively. Of all 814 patients, 157 patients (19.3%) underwent robotic gastrectomy and 308 (37.2%) were diagnosed with pathological stage II or III disease. Robotic gastrectomy was an independent positive predictor for recurrence-free survival in pathological stage II/III patients (hazard ratio: 0.56 [0.33-0.96], P = 0.035). Comparison of recurrence-free survival between robotic and laparoscopic approach using propensity score matching analysis verified that with less morbidity in the robotic group (P = 0.005).

Research conclusions

Age, ASA status, type of gastrectomy, and pathological T and N status were prognostic factors of minimally invasive gastrectomy for gastric cancer, and the use of a surgical robot may improve its long-term outcomes for advanced gastric cancer.

Research perspectives

Future studies to better prove the efficacy of robotic gastrectomy for advanced gastric cancer patients are warranted.

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ORIGINAL ARTICLE

Observational Study

Diagnostic usefulness of selected proteases and acute phase factors in patients with colorectal adenocarcinoma

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Abstract

BACKGROUND

Uncontrolled growth and loss of control over basic metabolic functions, leading to invasive proliferation and metastases, are the salient traits of malignant tumors in general and colorectal cancer in particular. Invasion and metastases hinder effective tumor treatment. While surgical techniques and radiotherapy can be used to remove tumor focus, only chemotherapy can eliminate dispersed neoplastic cells. However, the efficacy of the latter method is limited in the advanced stages of the disease. Therefore, recognition of the mechanisms involved in neoplastic cell spreading is indispensable for developing effective therapies.

AIM

To use a number of biomarkers involved in cancer progression and identify a panel that could be used for effective early diagnosis.

METHODS

We recruited 185 patients with colorectal adenocarcinoma (98 men, 87 women with median age 63). Thirty-five healthy controls were sex and age-matched. Dukes' staging was as follows: A = 22, B = 52, C = 72, D = 39. We analyzed patients' blood serum before surgery. We determined: (1) Cathepsin B (CB) with Barrett's method (fluorogenic substrate); (2) Leukocytic elastase (LE) in a complex

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with alpha 1 trypsin inhibitor (AAT) using the immunoenzymatic MERCK test; (3) Total sialic acid (TSA) with the colorimetric periodate-resorcinol method; (4) Lipid-bound sialic acid (LASA) with the colorimetric Taut's method; and (5) The antitrypsin activity (ATA) employing the colorimetric test.

RESULTS

In patients, the values of the five biochemical parameters were as follows: CB = 16.1 ± 8.8 mU/L, LE = 875 ± 598 μg/L, TSA = 99 ± 31 mg%, LASA = 0.68 ± 0.33 mg%, and ATA = 3211 ± 1504 U/mL. Except for LASA, they were significantly greater than those of controls: CB = 11.4 ± 6.5 mU/L, LE = 379 ± 187 μg/L, TSA = 71.4 ± 15.1 mg%, LASA = 0.69 ± 0.28 mg%, and ATA = 2016 ± 690 U/mL. For CB and LASA, the differences between the four Dukes' stages and controls were not statistically significant. The inter-stage differences for CB and LASA were also absent. The receiver operating characteristic (ROC) analysis revealed the potential diagnostic value of CB, TSA, and ATA. The area under ROC, sensitivity, and specificity for these three parameters were: 0.85, 72%, 90%; 0.75, 66%, 77%; and 0.77, 63%, 84%, respectively. The sensitivity and specificity for the three-parameter panel CB-TSA-ATA were equal to 88.2% and 100%, respectively.

CONCLUSION

The increased value of CB, TSA, and ATA parameters are associated with tumor biology, invasion, and metastasis of colorectal cancer. The presented evidence suggests the potential value of the CB-TSA-ATA biochemical marker panel in early diagnostics.

Key Words: Colorectal cancer; Cathepsin B; Acute phase reactants; Colorectal adenocarcinoma; Acute phase factor

Core Tip: We searched for biomarkers applicable to the early detection of colorectal adenocarcinoma. Five parameters were determined in sera of patients and healthy individuals: Cathepsin B activity, total sialic acids concentration, lipid-associated sialic acids concentration, elastase concentration, and alpha 1 antitrypsin activity. We performed receiver operating characteristic analysis for single and multiple parameters. While the sensitivity and specificity were not very high for single parameters, the combined analysis of cathepsin B, alpha 1 antitrypsin, and total sialic acids concentration yielded 88% sensitivity and 100% specificity. We believe that this set of markers can be useful in clinical practice.

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INTRODUCTION

The process of neoplastic invasion consists of two main stages: Penetration of tissues surrounding cancer and creation of metastases in places distant from the original location. During migration, cancer-transformed cells encounter anatomical barriers: The basement membrane and connective tissue. Cathepsins play an important role in the process of overcoming them.

Under normal conditions, cathepsins do not occur extracellularly or appear outside the cell only in small quantities. Their main function is to participate in processes related to the "turn-over" of endogenous proteins and degradation of exogenous proteins absorbed in the process of endocytosis[1-4]. In smaller concentrations, *e.g.*, in extralysosomal spaces, the enzymes can catalyze — by means of limited proteolysis — posttranslational processes of conversion of many peptides and proteins, including

growth factors and hormones such as albumins, insulins, endorphins, and enkephalins [5,6]. The release of cathepsins from cancer cells and their expression in the plasma membrane of cancer cells facilitates the dissolution of the basement membrane and participation in the proteolytic metastatic cascade. In clinical practice, a significant increase in the activity of cathepsin B (CB) is observed in the serum of patients with malignant tumors. This phenomenon occurs regardless of the location of the tumor and is closely related to the severity of ovarian, cervical, breast, laryngeal and colorectal cancer. It was also found that the activation of CB, which takes place with the participation of elastase coming from the neutrophil intumescence and tumor tissue, has an impact on its invasiveness and metastatic capacity. Studies have also confirmed that an increase in cathepsin expression in colorectal tumor tissue homogenates may be a sensitive marker for cancer progression[7-16].

Leukocytic elastase (LE) belongs to the group of serine proteases. It is located mainly in the azurophilic granules of neutrophils, where it is an active component of the phagocytic system along with other hydrolyses and reactive oxygen species. The enzyme is also cytochemically detected in the nuclear membrane, the Golgi complex, endoplasmic reticulum, and mitochondria[17,18]. It also participates in remodeling and tissue repair processes and modulates the activity of cytokines and their receptors (*e.g.*, mitogen-activated protein kinase 3). It can degrade elements of connective tissue by hydrolysis of elastin, various types of collagen, and other extracellular matrix proteins such as fibronectin, laminin, or proteoglycans. The physiological regulation of the activity and prevention of potentially destructive effects of elastase in pathological states is caused by protein inhibitors present in the blood serum. They include alfa 1 trypsin inhibitor (AAT), alpha 2-macroglobulin (alpha-2-MG), a secretory leukocytic protease inhibitor (SLPI), and elaphin[19-21]. In a healthy organism, LE remains in balance with them, and after the secretion from the cells, it is directly bound by inhibitors. The inhibitor molecules are thermally very stable and do not undergo proteolysis. Increased levels of LE in blood plasma, most often determined as LE-AAT complexes, have been found in many associated inflammatory conditions and are therefore considered acute-phase factors. Many authors regard the concentration of the above complexes to be a measure of the activity of the inflammatory process itself and be a marker of stimulation of neutrophils in the inflammatory focus. Lowering the level of AAT, caused by genetic or environmental factors, enables uncontrolled elastase activity and leads to many serious pathological conditions. This is caused by the enzyme-inhibitor imbalance and overexpression of the enzyme or a decrease in inhibitor concentration.

Sialic acid (N-acetyloneuraminic acid, NANA) is an organic compound from the sugar group, a derivative of neuraminic acid. It is a sugar component of glycoproteins and glycolipids. It occupies a terminal position in carbohydrate glycoprotein residues and has an important function in cell physiology as well as in the metabolism and maintenance of the proper concentration of glycoproteins in serum. It is also part of the ligand for the selectin and lectin receptors on leukocytes, T lymphocytes, platelets, and endothelium. It plays a key role in the immune response and hemostasis[22].

In the blood of patients suffering from metabolic disorders (*e.g.*, diabetes mellitus, atherosclerosis), a clear increase in the glycoprotein fraction is observed, while the sialic acid content remains low. In the case of cancer, the level of glycoproteins that contain normal or increased amounts of sialic acid increases in the blood. Increased sialisation is associated with the development of a neoplastic tumor and malignant cell metastasis. An increase in sialic acid concentration is observed in the case of malignant melanoma, lung, larynx, breast, ovary, prostate, liver, or colorectal cancer. It has been shown that the sialyltransferase activity in blood serum taken from people with cancer is increased and reaches its highest concentration at advanced stages of cancer development. The main areas of protein glycosylation include the endoplasmic reticulum and Golgi apparatus[23]. During the transformation of normal cells into cancer cells, there are significant differences in the biosynthesis of the sugar parts of proteins and membrane lipids. These changes are primarily of a qualitative nature. The external part of the plasma membrane of cancer cells has an increased number of sialic acid molecules compared to normal cells. Glycoproteins rich in sialic acids are more frequently found in case of metastatic cancer. The increased level of sialoglycoproteins and sialoglycolipids in neoplasms is mainly due to increased disintegration of cancer cells, increased synthesis and secretion of glycoconjugates (glycoproteins and glycolipids) containing sialic acid[24-32].

Recently, many works have been published on the activity of proteolytic enzymes and their inhibitors in blood serum. However, they do not solve all the problems related to the diagnostic and prognostic usefulness of these parameters in neoplastic diseases.

In this study, a statistically consistent group of colorectal cancer patients was collected: Women and men. The division of colorectal cancer into the colon and rectal cancer, histopathological criteria (adenocarcinoma), a clinical division system based on Dukes' cancer staging were considered.

The study presented below has both basic and diagnostic-clinical nature. The results of the study may be used in the future in the complementary diagnosis of colorectal adenocarcinoma to determine the severity of the disease and in further monitoring of patients under outpatient control and prognosis.

MATERIALS AND METHODS

Patients

One hundred and eighty-five patients were recruited from the Lower Silesian Oncology Center and the Provincial Specialist Hospital in Wroclaw. The study material presented in this paper was blood serum from patients with colorectal adenocarcinoma. The patients' blood serum was examined before surgery.

The examined patients were evaluated in terms of their age, sex, location of neoplastic lesions (colon, sigmoid colon, rectum), histopathological differentiation of neoplastic cells (G), and their clinical stages were based on the Dukes' classification. The characteristics of the examined patients are summarized in Table 1.

Biochemical measurements

The following tests were performed in blood serum according to the methods given below: (1) CB was determined with the use of fluorogenic substrate using the Barrett method[33]; (2) LE in a complex with AAT was determined immunoenzymatically using the MERCK test; (3) Total sialic acid (TSA) was determined colorimetrically using the periodate-resorcinol method, according to Jourdian *et al*[34]; (4) Lipid-bound sialic acid (LASA) was determined colorimetrically using Tautu *et al*[35]; and (5) The antitrypsin activity (ATA) in blood plasma (in the study referred to as antitrypsin capacity — ATA) was determined colorimetrically against trypsin using the method proposed by Warwas *et al*[36] and Dietz *et al*[37].

Statistical analysis

The examined continuous features were characterized by the distribution parameters of these features, *i.e.*, mean value (M), standard deviation (SD), and the number of patients (*n*). For the analysis of the statistical material, the following were used: For continuous features — single-factor analysis of variance (ANOVA), using Tukey's *post hoc* tests and multi-factor analysis of variance (MANOVA). The t-Student's *t*-test was also used for dependent samples. For features deviating from the normal distribution, which were also characterized by a median value, non-parametric tests were used: For independent samples — the Mann-Whitney *U* test, and for dependent samples — the Wilcoxon test. For categorized or dichotomous features, the χ^2 test and the non-parametric Kruskal-Wallis test were used. The relationship between these features was also studied by determining the Spearman correlation coefficient. The receiver operating characteristic (ROC) curve method was used to determine the threshold values of clinical markers (continuous variables) with optimal precision. The significance threshold *P* for all statistical tests was set to 0.05. The statistical analysis was conducted using Statistica v. 10.

RESULTS

Table 2 presents the results of biochemical parameters examined, their M, SD, *n*, including the group of colorectal cancer patients and the control group of healthy individuals.

In the group of patients with colorectal cancer, differences were observed in the values of examined biochemical parameters in blood serum compared to the control group. Despite clear changes in the levels and activity of the studied factors, not in all groups, these differences were statistically significant. Relevance was observed between patient groups suffering from colorectal cancers and the control group for CB, LE, TSA, and ATA.

Table 3 presents statistically significant differences between the examined groups of patients according to Dukes' classification and the control group. There were no

Table 1 Characteristics of patients with colorectal cancer

Number of patients with colorectal cancer	**185**
Age, median (age range)	63 (18-86)
Sex, *n* (%)	
Men	98 (55)
Women	87 (45)
Anatomical location, *n* (%)	
Colon	77 (42)
Sigmoid colon	37 (20)
Rectum	71 (38)
Histological differentiation of cells, *n* (%)	
G1	8 (4)
G2	103 (56)
G3	74 (40)
Division of patients according to Dukes' classification, *n* (%)	
A	22 (12)
B	52 (28)
C	72 (39)
D	39 (21)
Number of patients in the control group	**35**
Age, median (age range)	61 (19-85)
Sex, *n* (%)	
Men	19 (54)
Women	16 (46)

significant differences for CB and LASA ($P > 0.05$), while the differences for other parameters were: LE ($P < 0.001$), TSA ($P < 0.001$), ATA ($P < 0.01$).

Table 4 presents a comparison of statistically significant differences between individual parameters in the patient groups (A, B, C, D, according to Dukes' classification) and control group. There were statistically significant differences for all groups except for group A. Statistically significant differences between patient groups were also being observed. For LE, the most statistically significant difference was observed between groups C and D ($P < 0.002$). For TSA, the difference was most strongly pronounced for A and D ($P < 0.05$). There were no stage differences for ATA.

In Table 5 the correlations between in main parameters are summarized. The values of all the above-mentioned correlation coefficients were positive except for CB and ATA and LASA and ATA. The highest correlations were observed between CB and ATA; LE and TSA; TSA and ATA ($P < 0.001$).

No statistically significant differences in the examined parameters in respect to histopathological differentiation of tumor cells (G1, G2, G3) were observed (data not shown).

The examined biochemical factors in the blood serum were also examined using ROC analysis. The analyses are summarized in Figure 1. The highest diagnostic value was observed for a single value for CB in blood serum, with a threshold value of 11.22 mU/L. The sensitivity of the method was 72%, and its specificity was 90%. Then, respectively: for TSA, with a threshold value of 75.34 mg% — 66% and 77%; for LASA with a threshold value of 0.562mg% — 80% and 41%; for LE with a threshold value of 543 μg/L — 49% and 89% and ATA in blood serum with a threshold value of 2400 U/mL — 63% and 84%.

The analysis of the ROC curves of the two associated parameters has shown that the connection between CB and TSA gives a sensitivity of 73% and a specificity of 100%, with a value under the curve reaching 95%.

Table 2 Characteristics of the tested biochemical parameters in blood serum in all patients and in the control group

No.	Biochemical parameters tested	Groups	ABCD	K	Significance level *P* value
1	CB, mU/L	M	16.1	11.4	< 0.050
		n	185	35	
		SD	8.8	6.5	
2	LE, μg/L	M	875.1	379.1	< 0.001
		n	51	30	
		SD	597.9	187.3	
3	TSA, mg%	M	98.9	71.4	< 0.001
		n	71	31	
		SD	30.8	15.1	
4	LASA, mg%	M	0.68	0.69	NS
		n	68	35	
		SD	0.33	0.28	
5	ATA, U/mL	M	3211.4	2015.9	< 0.001
		n	74	29	
		SD	1504.1	689.6	

ABCD: All patients with colorectal adenocarcinoma; ATA: Antitrypsin activity; CB: Cathepsin B; K: Control group; LASA: Lipid-bound sialic acid; LE: Leukocytic elastase; M: Mean value; NS: Not significant; TSA: Total sialic acid.

Combining three parameters for the ROC curve: CB, TSA, and ATA, in blood serum, gives a sensitivity of 88% and a specificity of 100%, respectively, with a value of area under the curve of 95%.

In conclusion, ROC analysis has a high diagnostic value and can be helpful, especially in the combined analysis (biomarker panel determination).

DISCUSSION

The ability of the tumor to invade and metastasize is associated, among other pathogenic issues, with an increase in the expression of cysteine peptides. The source of these enzymes may be, in addition to the neoplastic tissue, neutrophils infiltrating the tumor. It is believed that CB determination may be helpful in the diagnosis and monitoring of colon and rectal cancer therapy[38-40].

In our study, the average CB activity determined in the blood serum of patients with colorectal cancers using a synthetic Z-Arg-Arg-N-MC substrate was about 1.5 times higher than that of healthy individuals ($P < 0.05$). Statistically significant results were obtained in the group of patients with different degrees of clinical advancement (ABCD: $P < 0.05$) (Table 2 and Figure 1A). On the other hand, CB did not statistically significantly differentiate patients according to Dukes' staging of colorectal cancer (A, B, C, D), although the average values in patients exceeded the average values in the control group (Table 3). Among other parameters, CB correlated only with ATA ($P < 0.001$) (Table 5).

The activity of CB in serums of patients with colon and rectal cancers was also studied by other authors. Dufek *et al*[41] observed a fivefold increase in this parameter. It was found that the CB activity in patients with colon and rectal cancers was significantly higher than in patients with polyps and in healthy individuals. This difference may be due to the use of the other substrate (Z-Ala-Arg-Arg-N-MC), which may also have been hydrolyzed by other proteases. These studies have also shown a high level of alkali-stable form of CB. In people with mild lesions (polyps) and in healthy people, the level of this form did not reach the threshold of determination. After the effective treatment, CB levels decreased significantly and increased again in case of metastases or resistant chemotherapy.

Table 3 Characteristics of biochemical parameters in patients' blood serum in relation to the severity according to the Dukes' classification

No.	Biochemical parameters tested	Groups	A	B	C	D	K	Significance level *P* value
1	CB, mU/L	M	19.9	15.4	16.8	15.5	11.4	NS
		n	22	52	72	39	35	
		SD	19.3	9.4	8.4	7.6	6.5	
2	LE, μg/L	M	386.1	929.9	602.1	1129.5	379.1	< 0.001
		n	5	16	15	15	30	
		SD	190.2	637.5	389.6	651.7	187.3	
3	TSA, mg%	M	67.8	100.8	92.9	107.2	71.4	< 0.001
		n	6	21	24	20	31	
		SD	7.4	39.3	25.1	26.3	15.1	
4	LASA, mg%	M	0.44	0.8	0.6	0.6	0.7	NS
		n	6	21	24	21	37	
		SD	0.12	0.51	0.23	0.20	0.31	
5	ATA, U/mL	M	3100.0	2925.0	3152.1	3576.2	2015.9	< 0.010
		n	2	21	24	21	29	
		SD	424.3	1294.5	1684.7	1548.8	689.6	

Dukes' classification system for colorectal cancer: A, B, C, D: All patients with colorectal adenocarcinoma; ATA: Antitrypsin activity; CB: Cathepsin B; K: Control group; LASA: Lipid-bound sialic acid; LE: Leukocytic elastase; M: Mean value; NS: Not significant; TSA: Total sialic acid.

Kos *et al*[40] also observed the fivefold increase in CB concentration using immune-enzymatic method. The CB level correlated well with stage C and D of Dukes' staging system. As in our research, no correlation between CB and age or gender was observed. However, it should be stressed that the antibodies used in this method detected both the active and non-active precursor form of this enzyme and their complexes with inhibitors, such as cystatins.

Similar studies on the proteolytic activity of blood serum with colon and rectal cancer were performed by Amiguet *et al*[42]. Similar to our research, the proteolytic activities of CB and elastase, were determined. Patients in Dukes' B and D stages were examined. The activities of the examined proteases were increased in relation to the control group of healthy individuals. In Dukes' stage D, the increase in CB levels was directly proportional to the weight of the tumor. In metastatic carcinomas, the increase in CB was accompanied by an increase in AAT concentration.

Padilla *et al*[43] showed, with the use of the immunoreactive method, that CB levels in colorectal cancer patients were different from the control group.

Clinical and pathological evaluation of patients with the use of serum CB and cathepsin D, based on the TNM system before and after the operation, was performed by Skrzydlewska *et al*[44]. The CB activity before the cancer tumor resection was significantly higher. However, in relation to the control group, both before and after the procedure, the CB activity was approximately 8.4 times lower. The authors concluded that the postoperative level of CB was associated with the involvement of the surrounding lymph nodes and higher when not accompanied by lymph node involvement. However, a relatively non-specific Z-Arg-pNA substrate was used to test the CB activity, and thus the observations might have been biased by the activity of other proteases.

Zore *et al*[45] examined CB levels in the complex with cystatin C using the ELISA method. Their observations show that CB in the Dukes' CD stage was significantly lower than in the AB stage (P = 0.02). Our study did not observe any significant differences between these groups (the results are not presented). The inhibitory capacity of cystatin C does not compensate for the increase in CB levels in patients suffering from colorectal cancers. This supports the hypothesis that inhibitory capacity might have been impaired during colorectal cancer progression.

Table 4 Statistical analysis of differences in the activity or concentration of examined parameters in serum of patients with adenocarcinoma and the control group

Patients' Groups					
LE	**A**	**B**	**C**	**D**	**K**
A	NS	NS	NS	NS	NS
B	NS	NS	$P < 0.05$	NS	$P < 0.001$
C	NS	$P < 0.05$	NS	$P < 0.002$	$P < 0.004$
D	NS	NS	$P < 0.002$	NS	$P < 0.001$
K	NS	$P < 0.001$	$P < 0.004$	NS	NS
TSA	**A**	**B**	**C**	**D**	**K**
A	NS	NS	NS	NS	NS
B	NS	NS	NS	NS	$P < 0.001$
C	NS	NS	NS	NS	$P < 0.003$
D	$P < 0.05$	NS	NS	NS	$P < 0.001$
K	NS	$P < 0.001$	$P < 0.003$	$P < 0.001$	NS
ATA	**A**	**B**	**C**	**D**	**K**
A	NS	NS	NS	NS	NS
B	NS	NS	NS	NS	$P < 0.02$
C	NS	NS	NS	NS	$P < 0.002$
D	NS	NS	NS	NS	$P < 0.001$
K	NS	$P < 0.02$	$P < 0.002$	$P < 0.001$	NS

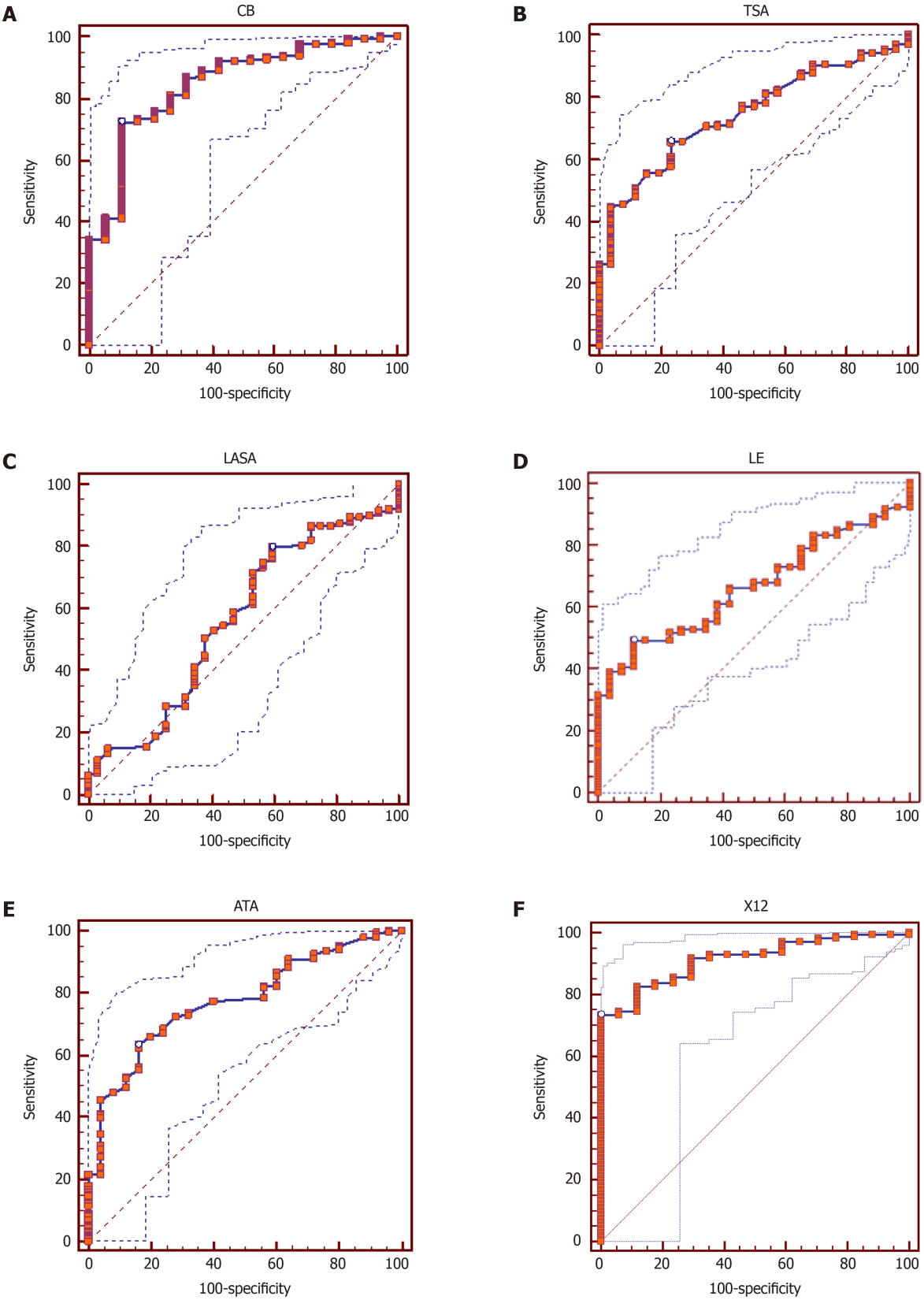
Significance levels of differences in the expected values of the analyzed biochemical parameters in patients divided into colorectal cancer patients (Dukes' stage A, B, C, D) and control group (K). The values of statistical significance — *P* were provided. A, B, C, D: All patients with colorectal adenocarcinoma; ATA: Antitrypsin activity; K: Control group; LE: Leukocytic elastase; NS: Not significant; TSA: Total sialic acid.

Table 5 Statistical analysis of correlations between the biochemical parameters examined in blood serum in all patients

Studied parameters	CB, mU/L	LE, μg/L	TSA, mg%	LASA, mg%	ATA, U/mL
CB, mU/L					-0.24 (187), $P < 0.001$
LE, μg/L			0.37 (140), $P < 0.001$	0.17 (141), $P < 0.05$	
TSA, mg%		0.37 (140), $P < 0.001$		0.19 (212), $P < 0.01$	0.33 (209), $P < 0.001$
LASA, mg%		0.17 (141), $P < 0.05$	0.19 (212), $P < 0.006$		-0.14 (210), $P < 0.05$
ATA, U/mL	-0.24 (187), $P < 0.001$		0.33 (209), $P < 0.001$	-0.14 (210), $P < 0.05$	

Values of linear correlation coefficients between concentrations or activities of the studied biochemical parameters (number of the examined). Description of other parts as in Table 2. ATA: Antitrypsin activity; CB: Cathepsin B; LASA: Lipid-bound sialic acid; LE: Leukocytic elastase; TSA: Total sialic acid.

Cysteine proteases — CB and cathepsin L levels in blood serum in patients with colorectal cancer were studied by Herszényi *et al*[46], who used the ELISA method for this purpose. CB correlated with the progressive Dukes' scale, reaching a 2.3 times higher level in patients compared to the control group. Analysis of the ROC curve confirmed the diagnostic importance of the examined factors, including CB. The sensitivity and specificity in the ROC analysis of CB were similar to the results obtained in our study (72 and 89% respectively *vs* 82 and 88%, and the areas under the ROC curve 0.85 *vs* 0.87), thus confirming the high diagnostic value of the studied parameter. Comparing CB with other biochemical parameters such as TSA and ATA in the ROC analysis results in an even higher level of sensitivity and specificity. Also, other researchers noticed that proteolytic enzymes are excellent indicators for colorectal cancers, often better than the commonly used tumor markers[47].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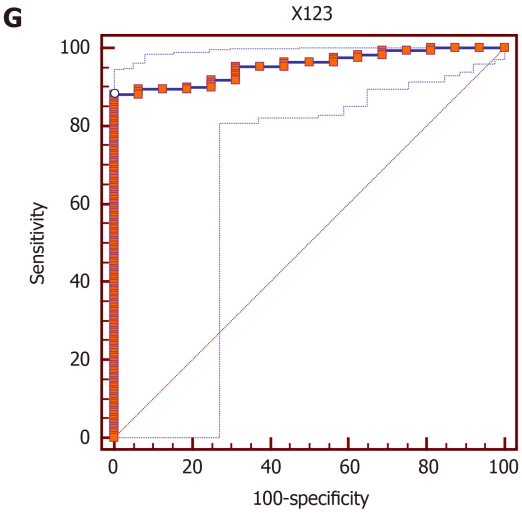

Figure 1 Diagram of receiver operating characteristic curve. A: For cathepsin B (CB). Threshold value of > 11.22 mU/L yielded sensitivity of 72.3% and specificity of 90%. Area under the receiver operating characteristic curve = 0.85; B: For total sialic acid (TSA). Threshold value > 75.34 mg% yielded sensitivity of 65.8% and specificity of 76.9%. Area under the receiver operating characteristic curve = 0.75; C: For lipid-bound sialic acid (LASA). Threshold value > 0.739 mg% yielded sensitivity of 79.7% and specificity of 40.6%. Area under the receiver operating characteristic curve = 0.56; D: For leukocytic elastase (LE). Threshold value > 543 μg/L yielded sensitivity of 49.2% and specificity of 88.5%. Area under the receiver operating characteristic curve = 0.56; E: For antitrypsin activity (ATA). Threshold value > 2400 U/mL yielded sensitivity of 63.2% and specificity of 84.0%. Area under the receiver operating characteristic curve = 0.77; F: For the combined two biochemical parameters. Threshold value > 0.4757 yielded sensitivity of 73.4% and specificity of 100%. Area under the receiver operating characteristic curve = 0.91; G: The three parameters combined (X123): CB, TSA, and ATA. Threshold value > 1.3457 yielded sensitivity of 88.2% and specificity of 100%. Area under the receiver operating characteristic curve = 0.95.

The imbalance of protease/inhibitors ratio is particularly relevant for LE and AAT involvement in the pathogenesis of cancer. The level of AAT, an acute-phase protein, increases in cancer patients in response to the increased levels of proteolytic enzymes released from leukocytes into circulation. In patients, despite an increase in their levels, the functional activity of the inhibitor decreases, thus disturbing the LE/AAT balance. This imbalance is further exacerbated by the fact that membrane forms of proteases, such as CB or elastase are more resistant to these inhibitors. Moreover, LE has itself ability to degrade those inhibitors[48]. A disturbed balance between LE and AAT may be associated with an increased risk of liver, cholecystitis, bladder, lymphoma, or lung cancer[49-55]. Apart from AAT, other protein inhibitors present in the blood serum are also responsible for the physiological regulation of LE: α2MG, SLPI, or elaphin. SLPI, as it is clear from the work by Sugino *et al*[54], performed in various types of cancer, including colorectal cancer, has a dual effect. On the one hand, it suppresses the invasion of neoplastic cells, and on the other hand, it promotes metastasis that transmits through blood circulation.

The inclusion of serum ATA and LE, as indicated by the results of our research, may be a factor informing about the balance of serine proteases.

LE in all listed patient groups according to the Dukes' classification: Combined ABCD stages and A, B, C, D stages separately (Table 2 and Figure 1D), shows a clear statistically significant difference when compared to the control group. Its activity reached the highest values in groups B and D. It is possible that it is associated with subsequent stages of cancer spread and the presence of metastatic foci rich in granulocytic intumescence, increased elastase-induced adhesion of cancer cells to the endothelium of vessels, or through mitogenic activity[56,57].

The different activity of serum elastase, in successive stages of the disease according to the Dukes' classification eliminates the possibility of using this parameter for early diagnosis in colorectal cancers. In addition, serum elastase is characterized by relatively low sensitivity in the ROC analysis (49%) (Figure 1D). On the other hand, some authors have found it as a putative diagnostic biomarker and also a potential therapeutic target[58].

AAT is a blood plasma protein belonging to α_1-globulin fraction, one of the strongest inhibitors of circulating serine proteases (serpins). It is also an acute-phase protein, synthesized mainly in the liver but also by macrophages. AAT has the ability to inactivate many proteolytic enzymes, but its most important effect is the inactivation of LE released by neutrophils as a result of an inflammatory reaction.

In our study, statistically significant ($P < 0.001$) elevated ATA levels were observed in the cumulative group of patients, regardless of the Dukes' classification stage (ABCD) in relation to the control group. In the B, C, and D stages, in relation to the control, these levels were highly statistically significant ($P < 0.001$; $P < 0.004$; $P < 0.001$). Also, ATA differentiated very well patients with Dukes' stages B against C and C against D ($P < 0.05$ and $P < 0.002$, respectively). In our ROC analysis, with the cut-off value > 2400 U/mL, the ATA value reached the sensitivity of 63% and specificity of 84%, respectively. The listed sensitivity and specificity parameters are lower than in the ROC analysis for CB (72% and 90%, respectively).

There is little clinical work on the contribution of AAT to the diagnosis or monitoring of the treatment of colorectal cancer[59-64]. Yüceyar *et al*[65] and Gallardo-Valverde *et al*[66] did not find relationship between the AAT and the severity of colorectal cancer and showed a statistically significant correlation between AAT and other biochemical factors such as acute-phase protein, carcinoembryonic antigen (CEA), or tumor-associated trypsin inhibitor (TATI).

Bernacka *et al*[67] studied plasma levels of AAT in patients with gastrointestinal cancers: Stomach and colorectal cancers of adenocarcinoma type. This marker did not differentiate colorectal cancer patients in terms of local and metastatic lesions. However, it had the highest level in stage C and differentiated patients in terms of the histological degree of tumor stage (G). The author postulates that AAT levels are associated with increased production by liver cells in response to the increased release of lysosomal proteases of tumor cells or from mononuclear inflammatory tumor-infiltrating cells. It seems that tumor cells may be the third source of antiproteases.

Ward *et al*[68], using proteomic profiling, identified AAT as having the potential to classify the colorectal patients with 95% sensitivity and 91% specificity.

Interesting conclusions were drawn from the work of Bujanda *et al*[64], who studied a group of 42 colorectal cancer patients using combined AAT, matrix metalloproteinase 7 (MMP-7), urokinase-type plasmin activator receptor (uPAR), and cyclooxygenase-2 (COX-2). Compared to the control group, AAT levels were about 1.4 times higher at stages B and C, and the AAT level was high and reached its value under the ROC curve (0.88). The above results are similar to those obtained in our study. The level under the ROC curve was 0.77. In patients in stages B and C, ATA levels were 1.5 and 1.6 times higher, respectively, than in the control group. AAT has a promising diagnostic profile and, most importantly, at the early stages of colorectal cancer.

The neoplastic process is associated not only with the activation of the cascade system of proteolytic enzymes, the activation of which mainly takes place with the participation of CB and LE, but also induces the activation of acute-phase proteins.

The interest in the sialic acids (TSA, LASA) as markers useful in diagnosing and monitoring the course of many diseases, including colorectal cancer, is reflected in the publications briefly reviewed in[69-72]. Increased levels of sialic acid expression are associated with changes in biosynthesis and posttranslational processes of acute-phase proteins glycosylation in the liver. This phenomenon is associated with increased expression of sialyltransferases by cancer cells[73]. The mechanism of increased TSA levels in serum takes the following into account: (1) Spontaneous release of the compounds from the surface of cancer cells; (2) Increase in concentration and/or glycosylation of serum glycoproteins; and (3) Secondary inflammatory reaction associated with the increase in acute-phase proteins[32,74]. Increased sialyltransferases activities, observed in cancer cells, results in increased glycoprotein secretion as well as the secretion of cell membrane components into the culture medium. The cancer cell hypoxia may also contribute to the above[75]. Increased sialisation of glycosphingolipids leads to abnormal adhesion and a disturbed premembranous signal exchange [75]. Determination of sialic acids is a laboratory marker of many pathological lesions. A significant increase in serum levels of sialic acids was observed in many malignant diseases[76]. Elevated levels of TSA or LASA were observed in malignant melanoma, lung, breast, ovary, and laryngeal cancers[28,32,77], as well as in colorectal cancer[26,31,70,78-82].

In our study, TSA in blood serum was elevated and statistically significantly different from the control group. The above observation concerns the cumulative group of patients (ABCD: $P < 0.001$) as well as individual groups (B, C, D in relation to the control: $P < 0.001$; $P < 0.003$; $P < 0.001$ respectively). The lowest level among patients was found in group A, and the highest in group D. The above clearly shows differences between particular patient groups. TSA was only less effective than ATA. The TSA level moderately but statistically significantly correlates with LE and ATA. The results of the ROC analysis are interesting. With a cut-off value for TSA > 75.34 mg%, the sensitivity of the method was 66%, and its specificity was 77%. In ROC analysis TSA clearly benefits when combined CB with the same cut-off values)

sensitivity 74% and specificity 100%were obtained. The sensitivity further increases if we also take the ATA into account, which is also studied by the authors and reflects the AAT level. In the TSA test, the main component determined is the sialic acid associated with proteins. The level of sialic acids increases in the sera of cancer patients as a result of increased concentration of acute-phase proteins rather than gangliosides from decaying cancer cells[65,74,75,83].

CONCLUSION

We have found a statistically significant increase in the CB activity in the blood serum of the examined individuals suffering from colorectal cancers. The highest CB level was observed in Dukes' stage A patients, and in stages B, C and D, it was lower and comparable to each other.

Concordantly, the following increased concentrations in blood serum of investigated markers were observed: LE, TSA, and ATA. According to Dukes' classification, these values were statistically significantly increased with respect to the control group, gradually rising from the A to D stage.

The ROC analysis showed the high diagnostic value of CB, TSA, ATA determinations in blood serum, both in the single and combined analysis (biomarker panels) with two biochemical parameters: CB and TSA, and with three parameters: CB, TSA, and ATA. The above results suggest high diagnostic usefulness in determining these 2 or 3 combined parameters in relation to single determinations, obtaining sensitivity and specificity of 88.2% and 100% for three parameters.

ARTICLE HIGHLIGHTS

Research background

Recognition of the mechanisms involved in neoplastic cell spreading is indispensable for the early diagnosis and detection of colorectal cancer.

Research motivation

Colorectal cancer is the third most common type of cancer, making up about 10% of all cases. In 2018, there were 1.09 million new cases and 551000 deaths from the disease. Consequently, early diagnosis of colorectal cancer remains a significant medical and economic problem.

Research objectives

Using several biomarkers involved in cancer progression, we have tried to identify a panel that could be used for effective early diagnosis.

Research methods

Before surgery, we analyzed the blood serum of 185 patients with colorectal cancer and determined: Cathepsin B (CB), leukocytic elastase (LE), total sialic acid (TSA), lipid-bound sialic acid (LASA), and antitrypsin activity (ATA).

Research results

The receiver operating characteristic analysis revealed the potential diagnostic value of CB, TSA, and ATA. The sensitivity and specificity for the three-parameter panel CB-TSA-ATA were equal to 88.2% and 100%, respectively.

Research conclusions

The increased value of CB, TSA, and ATA parameters are associated with tumor biology, invasion, and metastasis of colorectal cancer.

Research perspectives

The presented evidence suggests the potential diagnostic and prognostic value of the CB-TSA-ATA biochemical marker panel.

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ORIGINAL ARTICLE

Observational Study

Impact of a colorectal cancer screening program implantation on delays and prognosis of non-screening detected colorectal cancer

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Abstract

BACKGROUND

The implementation of a colorectal cancer (CRC) screening programme may increase the awareness of Primary Care Physicians, reduce the diagnostic delay in CRC detected outside the scope of the screening programme and thus improve prognosis.

AIM

To determine the effect of implementation of a CRC screening programme on diagnostic delays and prognosis of CRC detected outside the scope of a screening programme.

METHODS

We performed a retrospective intervention study with a pre-post design. We identified 322 patients with incident and confirmed CRC in the pre-implantation cohort (June 2014 – May 2015) and 285 in the post-implantation cohort (June 2017 - May 2018) in the Cancer Registry detected outside the scope of a CRC screening programme. In each patient we calculated the different healthcare diagnostics delays: global, primary and secondary healthcare, referral and colonoscopy-related delays. In addition, we collected the initial healthcare that evaluated the patient, the home location (urban/rural), and the CRC stage at diagnosis. We

determined the two-year survival and we performed a multivariate proportional hazard regression analysis to determine the variables associated with survival.

RESULTS

We did not detect any differences in the patient or CRC baseline-related variables. A total of 20.1% of patients was detected with metastatic disease. There was a significant increase in direct referral to colonoscopy from primary healthcare (25.5%, 35.8%; P = 0.04) in the post-implantation cohort. Diagnostic delay was reduced by 24 d (106.64 ± 148.84 days, 82.84 ± 109.31 d; P = 0.02) due to the reduction in secondary healthcare delay (46.01 ± 111.65 d; 29.20 ± 60.83 d; P = 0.02). However, we did not find any differences in CRC stage at diagnosis or in two-year survival (70.3%; P = 0.9). Variables independently associated with two-year risk of death were age (Hazard Ratio-HR: 1.06, 95%CI: 1.04-1.07), CRC stage (II HR: 2.17, 95%CI: 1.07-4.40; III HR: 3.07, 95%CI: 1.56-6.08; IV HR: 19.22, 95%CI: 9.86-37.44; unknown HR: 9.24, 95%CI: 4.27-19.99), initial healthcare consultation (secondary HR: 2.93, 95%CI: 1.01-8.55; emergency department HR: 2.06, 95%CI: 0.67-6.34), hospitalization during the diagnostic process (HR: 1.67, 95%CI: 1.17-2.38) and urban residence (HR: 1.44, 95%CI: 1.06-1.98).

CONCLUSION

Although implementation of a CRC screening programme can reduce diagnostic delays for CRC detected in symptomatic patients, this has no effect on CRC stage or survival.

Key Words: Colorectal cancer; Population based screening; Primary healthcare; Diagnostic delay; Prognosis

Core Tip: We have designed a retrospective intervention study with a pre-post design to confirm the hypothesis that the implementation of a colorectal cancer (CRC) screening program may increase the awareness of primary care physicians and, thus, reduce the diagnostic delays in CRC detected outside the screening program and improve prognosis. Our results confirm that the implementation of the CRC screening program reduced the diagnostic delays due to an increase in the direct referrals to colonoscopy from primary healthcare. However, this reduction in the delays had no effect on the stage at diagnosis or in the two year survival. These later results were confirmed in a multivariable Cox regression analysis.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most important health problems in the Western world. In 2018, almost half a million new cases were diagnosed in Europe and 250,000 patients died due to CRC[1]. In order to reduce the disease burden, population-based CRC screening programmes have been established in the Western world. This strategy has demonstrated its efficacy to reduce CRC mortality and incidence in randomized controlled trials. Furthermore, we have real data showing that implementation of CRC screening programmes has achieved its expected efficiency in reducing both CRC mortality and incidence[2,3].

In spite of the implementation of CRC screening programmes, most CRC are detected among symptomatic patients outside the scope of CRC screening mainly due to the limited participation and the detection in age cohorts that are not candidates for CRC screening[4,5]. However, as in breast cancer screening, the implementation of

conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Galicia, Spain (code 2016/274). As long as the study was based on database use, no informed consent was required. The information was accessed according to prevailing European and Spanish legislation.

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

Conflict-of-interest statement: There are no conflicts of interest to report.

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CRC screening may have an additional positive effect on these patients due to increased awareness and creation of multidisciplinary teams[6]. In this sense, CRC screening may increase the CRC awareness of patients and primary care physicians (PCPs) and promote use of faecal immunochemical test (FIT) as a triage test to refer patients to colonoscopy[7].

The delay to diagnosis in cancer is due to factors related to the patient and health system. The period from initial symptoms until final diagnosis is made can be highly variable. Although the common belief is that a longer delay can lead to an advanced stage at diagnosis and worse prognosis, evidence on CRC is controversial[8]. Patients seeking assistance with more severe symptoms are diagnosed in a shorter period and have more advanced disease[9]. In contrast, there is no evidence that a health system delay lower than six months worsens prognosis in the context of an outpatient diagnosis[10].

Based on the hypothesis that implementation of a mass CRC screening programme could raise awareness of patients and PCPs, we decided to design a retrospective intervention study to determine whether implementation of a CRC screening programme could reduce health system delays and, secondarily, improve CRC staging at diagnosis and long term survival.

MATERIALS AND METHODS

Study design

We designed a retrospective intervention study with a pre-post design without a control group.

Description of the intervention

The intervention was the first round of the Galician CRC screening programme that took place between 1 July 2015 and 30 June 2017 in Ourense, Spain. Galician CRC mass screening is based on biennial FIT with a 20 μg haemoglobin/g of faeces threshold. FIT is offered to subjects aged 50 to 69 years. It is coordinated by the Public Health Department of the Galician Regional Health Department. They are in charge of the identification of subjects, invitation to participate, reception of FIT results, citation of patients with a positive result to perform a colonoscopy and final evaluation of the endoscopic and histological results. Primary healthcare clinics are in charge of promoting participation in the screening programme, collecting FIT kits and evaluation of subjects with a positive FIT prior to colonoscopy. The hospitals in each health area are responsible for FIT analysis, colonoscopies, histological analysis and evaluation and treatment of patients with a CRC. Finally, personnel at the Coordination Unit key in data into the screening programme's information system regarding CRC stage according to the AJCC classification,[11]. the final classification of patients with a positive result[12]. as well as several quality endoscopist indicators according to the Spanish guideline on quality in screening colonoscopy[13]. During the implantation of the CRC screening program no change was performed in the diagnostic pathways for CRC diagnosis in symptomatic patients.

Inclusion criteria and definition of the cohorts

Pre cohort: We included all invasive incident CRC histologically confirmed detected in the natural year before implementation of the CRC screening programme (1 July 2014 – 30 June 2015) in Ourense.

Post cohort: We included all invasive incident CRC histologically confirmed and detected outside the scope of the CRC screening programme in the natural year after the first round: (1 July 2017- 30 June 2018).

Identification of the incident CRC

We identified the incident using the case identification structure developed and validated by the project for implementation of the Galician Tumour Registry (Project REGAT). REGAT uses the topographic codes ICD-O-3.1 C18-C19-C20 to identify the CRC[14]. Codes C18.1 (appendix), C21 (anus and anal canal) were excluded. REGAT data were crosslinked with the Galician CRC screening information system to exclude those patients with a CRC diagnosed within the screening programme.

Variables analyzed

We collected information regarding: (1) Demographics (age and sex); and (2) Tumour

location in relation to the splenic flexure: proximal (caecum, ascending colon, hepatic flexure and transverse colon) and distal (rectum, sigma, descending colon and splenic flexure).

Cancer stage at diagnosis according to the TNM classification (AJCC 7th edition) [11]. We used the following data to determine the stage at diagnosis: clinical or anatomo-pathological stage for metastatic disease, imaging tests for the local rectal cancer stage (T and N), anatomo-pathological evaluation for the remaining situations (colon cancer T and N).

We searched in IANUS, the unified clinical record database of the Galician Health Department, for information regarding the contacts and referrals in the healthcare system. IANUS includes all information regarding attendance in primary and secondary healthcare as well as emergency departments and hospitalization. We determined the first contact in the health system (primary, secondary, emergency), whether the patient required hospitalization during the diagnostic process and the diagnostic delays. We defined five diagnostic delays (Figure 1): (1) Global diagnostic delay: Overall delay from the first consultation to definitive diagnosis; (2) Primary healthcare delay: Delay from the initial evaluation in primary healthcare until the decision to refer to secondary healthcare. In the event of colonoscopy being directly requested from primary healthcare, this date was considered as the referral date; (3) Referral delay: Delay from the primary healthcare referral to the first attendance in secondary healthcare (either clinical consultation or performing of colonoscopy); (4) Secondary healthcare delay: Delay from the first attendance in secondary healthcare to final diagnosis; and (5) Colonoscopy delay: Delay from the colonoscopy request to the performing of colonoscopy.

Statistical analysis

First, we performed a descriptive analysis of the variables included: number and frequencies in the qualitative variables and mean and standard deviation in the quantitative variables. We determined whether there were differences between both cohorts in the diagnostic pathways (hospitalization, direct referral to colonoscopy from primary healthcare) using the Chi-square test. In order to detect whether there were differences in the referral delays between both cohorts we used the Student *t* test. We analyzed whether there were differences in two-year survival between both cohorts in the Kaplan-Meier analysis using the log-rank test. Finally, to control confounding variables we performed a Cox multivariate regression analysis and we determined which variables were independently associated with survival after diagnosis. The study was statistically reviewed by a biomedical statistician.

Ethics aspects

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Galicia, Spain (code 2016/274). As long as the study was based on database use, no informed consent was required. The information was accessed according to prevailing European and Spanish legislation.

RESULTS

Description of the sample

We identified records from 757 patients in the two periods analyzed in the cancer registry. We excluded 92 patients that did not meet the inclusion criteria and 58 patients with CRC detected within the CRC screening programme in the post-implantation cohort. Finally, the pre-implantation and post-implantation cohort consisted of 322 and 285 patients, respectively (Figure 2).

As we show in Table 1, we did not detect baseline differences between both cohorts. CRC was detected more commonly in males (59.6%) with a mean age of 74.5 ± 11.5 years and more than two thirds were distal to the splenic flexure. There were no differences with respect to the place of residence either. Most patients were initially evaluated in primary healthcare but up to 41.0% required hospitalization before reaching final diagnosis. Diagnosis was made through colonoscopy in 89.8% of detected CRC. In this sense, we detected a significant increase in the colonoscopy directly requested from primary healthcare in the post-implantation cohort (P = 0.04). When we limited the analysis to those patients initially seen in primary healthcare (522), the results were similar. In this sense, we only found differences in the rate of colonoscopy directly referred from primary healthcare (29.2%, 42.3%; P = 0.005).

Table 1 Characteristics of the patients included

	Pre-implantation cohort (*n* = 322)	Post-implantation cohort (*n* = 285)	*P* value[1]
Sex			0.1
Male	184 (57.1%)	178 (62.5%)	
Female	138 (42.9%)	107 (37.5%)	
Age (yr)	74.1 ± 11.8	74.8 ± 11.1	0.4
Colorectal location			0.8
Distal to splenic	221 (68.6%)	197 (69.1%)	
Proximal to splenic	101 (31.4%)	88 (30.9%)	
TNM			
I	45 (14.0%)	47 (16.5%)	
II	93 (26.9%)	71 (24.9%)	
III	101 (31.4%)	99 (34.7%)	0.5
IV	65 (20.2%)	57 (20.0%)	
Unknown	18 (5.6%)	11 (3.9%)	
Rural/Urban			
Rural	218 (67.9%)	195 (68.4%)	0.8
Urban	103 (32.1%)	90 (31.6%)	
Initial consultation			
Primary healthcare	281 (87.3%)	241 (84.6%)	
Secondar y healthcare	33 (10.2%)	37 (13.0%)	0.5
Emergency department	8 (2.5%)	7 (2.5%)	
Hospitalization			
Yes	135 (41.9%)	114 (40.0%)	0.6
No	187 (52.2%)	171 (60.0%)	
Colonoscopy request			
Primary healthcare	82 (25.5%)	102 (35.8%)	
Secondary healthcare			0.04
After referral	160 (49.7%)	116 (40.7%)	
Direct request	45 (14.0%)	40 (14.0%)	
No colonoscopy	35 (10.9%)	27 (9.5%)	

[1]Statistical significance in the univariate analysis using the Chi-square test for qualitative variables and the Student *t* test for quantitative variables.

Delay to diagnosis

The delay to diagnosis was reduced in 24 d after implantation of the CRC screening programme (P = 0.02). We did not detect any differences in the primary healthcare, referral or colonoscopy delay. The reduction was due to a secondary healthcare delay in relation to an increased rate of direct referral to colonoscopy from primary healthcare, as we show in Table 2 and Figure 3.

The global delay was also reduced by 27 d in patients evaluated initially in primary healthcare (117.66 ± 154.08 days, 90.06 ± 111.31 days; P = 0.02) also due to a reduction in secondary healthcare delay (48.17 ± 116.42 d, 26.89 ± 54.50 d; P = 0.02). There were no differences in primary healthcare, referral or colonoscopy delay.

Factors associated with survival

The incidence of metastatic CRC remained stable (20.1%) in both cohorts and overall survival after one and two years was 71.3% and 70.3% without differences in the log-

Table 2 Delay to colorectal cancer diagnosis in the pre and post-implantation cohorts

	Pre-implantation cohort (*n* = 322)	Post-implantation cohort (*n* = 285)	*P* value[1]
Global diagnostic delay (d)	106.64 ± 148.84	82.84 ± 109.31	0.02
Primary healthcare delay (d)	35.88 ± 84.47	39.28 ± 98.03	0.7
Referral delay (d)	13.18 ± 25.77	16.02 ± 41.63	0.4
Secondary healthcare delay (d)	46.01 ± 111.65	29.20 ± 60.83	0.02
Colonoscopy delay (d)	43.71 ± 78.22	37.75 ± 53.37	0.3

[1]Statistical significance in the univariate analysis using the Student *t* test.

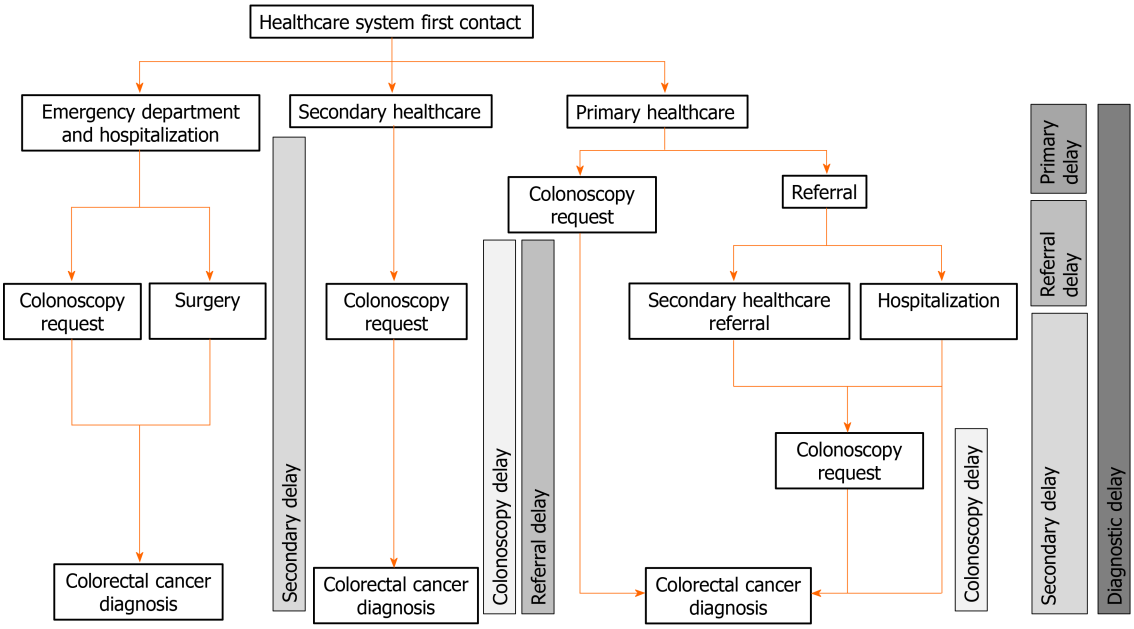

Figure 1 Flowchart of the referral and diagnostic pathways.

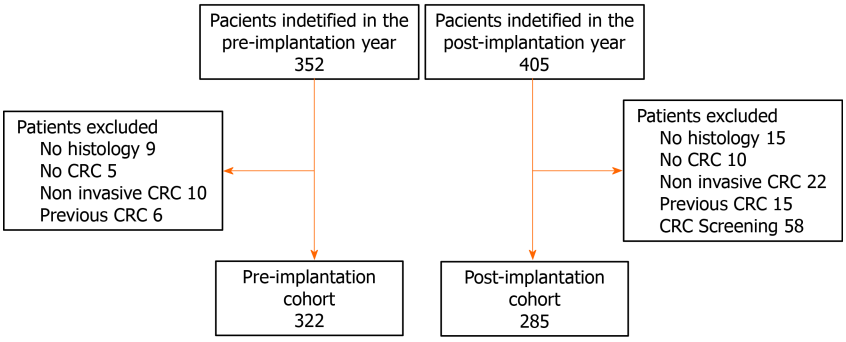

Figure 2 Flowchart of the patients included in the analysis.

rank test (P = 0.9) as we show in Figure 4. These results were confirmed in the Cox multivariate regression analysis and there were no differences in the survival between both cohorts (post-implantation cohort HR: 1.12, 95%CI: 0.83-1.51). As we show in Table 3, only age, CRC staging according to TNM classification, initial healthcare consultation, hospitalization during the diagnostic process and residence were independently associated with death after CRC diagnosis.

Table 3 Factors associated with survival

	Hazard ratio[1] (95%CI)
Sex	
Male	1
Female	1.18 (0.89-1.58)
Age (yr)	1.06 (1.04-1.07)
Colorectal location	
Distal to splenic	1
Proximal to splenic	0.84 (0.62-1.13)
Cohort	
Pre-implantation	1
Post-implantation	1.12 (0.83-1.51)
TNM	
I	1
II	2.17 (1.07-4.40)
III	3.07 (1.56-6.08)
IV	19.22 (9.86-37.44)
Unknown	9.24 (4.27-19.99)
Initial consultation	
Primary healthcare	1
Secondary healthcare	2.93 (1.01-8.55)
Emergency department	2.06 (0.67-6.34)
Hospitalization	
Yes	1.67 (1.17-2.38)
No	1
Colonoscopy request	
Primary healthcare	1.79 (0.96-3.35)
Secondary healthcare	1.54 (0.92-2.58)
After referral from Primary Healthcare	0.74 (0.25-2.21)
Direct request	1
No colonoscopy	
Rural/Urban	
Rural	1
Urban	1.44 (1.06-1.98)
Diagnostic delay (d)	1.001 (1.00-1.002)

[1]Hazard Ratio and its 95% confidence interval calculated using a multivariate proportional hazard regression analysis.

DISCUSSION

Our study shows that implementation of the CRC screening programme reduced healthcare referral delays due to the direct request of colonoscopy from primary healthcare. Unfortunately, this reduction in referral delay had no effect on CRC staging at diagnosis nor on the two-year survival. Finally, although we detected several variables associated with overall survival, multivariate logistic analysis confirms that neither implantation of the CRC screening nor diagnostic delay were related to the prognosis of CRC detected outside the scope of CRC screening.

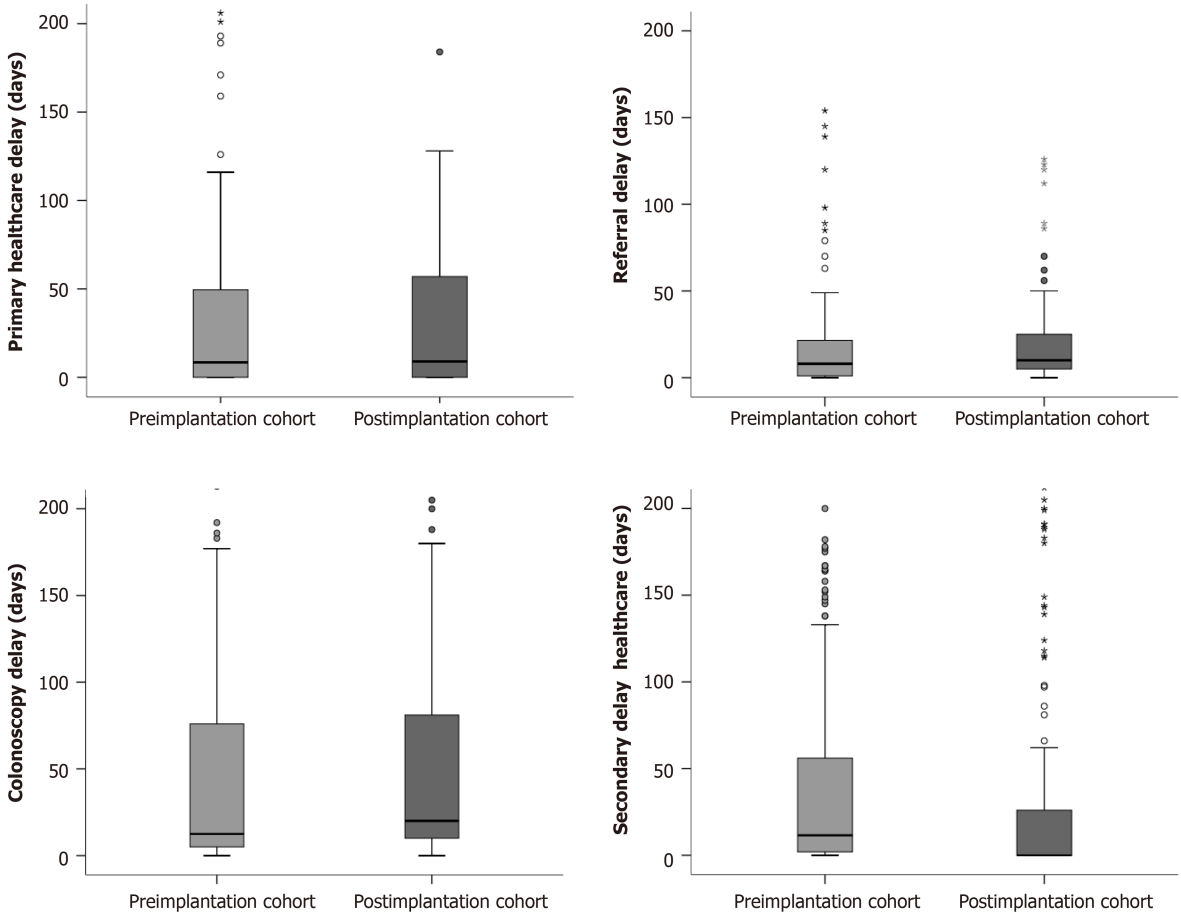

Figure 3 Healthcare diagnostic delays. We show the distribution of the primary and secondary healthcare, referral and colonoscopy delays expressed in days.

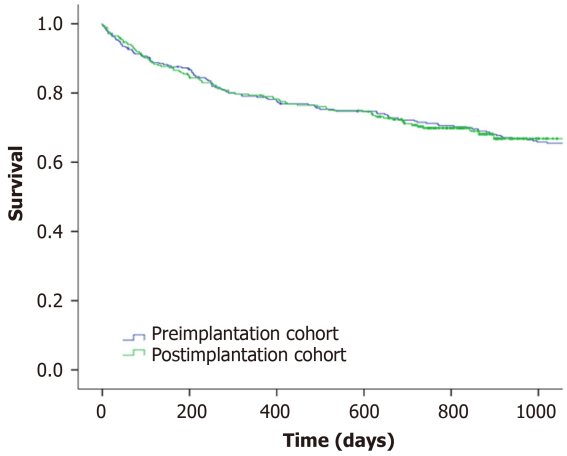

Figure 4 Survival curves of the pre and post-implantation cohorts. Survival curves were calculated using the Kaplan-Meier method.

PCPs play an important role in CRC care, from encouraging screening and accurate diagnosis to providing care during and after treatment for cancer and any comorbid complications. The implication of PCPs on CRC screening is variable according to the screening programme. Participation rates are increased when PCPs are involved in the invitation process. However, in the European population-based programmes in Europe PCPs play a rather supportive, informative or facilitating role[15]. In our case, PCPs receive full information on the screening programme organization and they are in charge of promoting participation as well as resolving any doubts. Within the training, PCPs are reminded which symptoms may lead to suspicion of CRC as well as the established referral pathways, including direct referral criteria for colonoscopy evaluation from primary healthcare[8,16].

We designed this analysis under the hypothesis that increased awareness on CRC and training in the diagnosis of CRC and the established protocols could reduce delays attributed to the health system. In this sense, our results confirm that implementation of the screening programme enabled a reduction in the diagnostic delay due to an increase in direct referrals to colonoscopy from primary healthcare. PCPs, as demonstrated in our study, are the main gateway and responsible for a significant part of the delay[17-19]. The role of PCPs in CRC diagnosis is complex since gastrointestinal symptoms that may suggest CRC are very common, the CRC prevalence low and the diagnostic performance of available symptom-based tools is very limited[20]. Recently, implementation of the faecal immunochemical test to triage patients with gastrointestinal symptoms in primary healthcare has improved diagnostic referral pathways[20,21]. In the health area of Ourense, faecal immunochemical test was implemented as a triage test seven years ago, so we cannot attribute the decreases in delay to this modification[16].

However, it is relevant that, despite the reduction in delay, we have not detected any changes in the stage at diagnosis or in the prognosis of CRC. These data are in accordance with results previously published by our group[8]. and with the data in the available meta-analysis on the effect of diagnostic delays in CRC prognosis[9]. In this sense, the prognosis of patients with shorter diagnostic delay is worse due to presentation with urgent symptoms that require hospitalization or more serious systemic symptoms[19]. In fact, in our study, hospitalization during admission was associated with a higher risk of mortality after diagnosis. In our research, although initial urgent presentation was rare, up to 40% of patients required hospitalization during the diagnostic process, similar to the information available on literature[22-24]. This lack of relationship between delay and prognosis may be related to different forms of presentation. In this sense, a prospective study on patients that met the National Institute for Health and Care Excellence referral criteria demonstrated that a delay of more than six months was associated with a worse prognosis compared to patients with the same symptoms diagnosed in an interval of less than one month[10].

Our study has two main strengths. We had the opportunity to evaluate the effect of the CRC screening programme on the diagnostic delays of CRC detected in symptomatic patients. This is the first study that evaluates additional impacts of the implementation of CRC screening on CRC diagnosis. No study has evaluated whether a CRC screening programme can increase the awareness of patients and PCPs, reduce delays and improve prognosis. However, we could identify all the CRC through the Galician cancer registry, confirm the diagnosis in IANUS, the centralized clinical record and determine when the patient was evaluated in the health system and thus calculate all the referral delays[25].

There are several limitations. Due to the design of the study, we could not evaluate the effect of CRC screening on the patient delays to seek assistance. Patient delay accounts for a relevant proportion of the delay between the onset of symptoms and the final diagnosis[26]. Moreover, we did not collect the initial symptoms as long as they were not collected uniformly in the clinical records.

CONCLUSION

To conclude, the implementation of a CRC screening programme enabled reduction of health system diagnostic delay by means of increased patients referred directly by PCPs to colonoscopy. However, this reduction in referral delay did not modify either CRC stage at diagnosis or two-year survival.

ARTICLE HIGHLIGHTS

Research background

In spite of the implementation of colorectal cancer (CRC) screening programmes, most CRC are detected among symptomatic patients outside the scope of CRC screening. However, they may increase the CRC awareness of patients and primary care physicians (PCP).

Research motivation

The implementation of a mass CRC screening programme could raise awareness of patients and PCPs, we decided to design a retrospective intervention study to

determine whether implementation of a CRC screening programme could reduce health system delays and, secondarily, improve CRC staging at diagnosis and long term survival.

Research objectives

To determine the effect of implementation of a CRC screening programme on diagnostic delays and prognosis of CRC detected outside the scope of a screening programme.

Research methods

We designed a retrospective intervention study with a pre-post design without a control group. We compared diagnostic delays, CRC stage and two year survival of a yearly CRC diagnosed before the implementation of a CRC screening programa with a CRC cohort diagnosed the year after the first round.

Research results

There was a significant increase in direct referral to colonoscopy from primary healthcare (25.5%, 35.8%; $P = 0.04$) in the post-implantation cohort. Diagnostic delay was reduced by 24 d (106.64 ± 148.84 d, 82.84 ± 109.31 d; $P = 0.02$) due to the reduction in secondary healthcare delay (46.01 ± 111.65 d; 29.20 ± 60.83 d; $P = 0.02$). However, we did not find any differences in CRC stage at diagnosis or in two-year survival (70.3%; $P = 0.9$).

Research conclusions

Although implementation of a CRC screening programme can reduce diagnostic delays for CRC detected in symptomatic patients, this has no effect on CRC stage or survival.

Research perspectives

We need more research on the motivations and perspectives of patients seeking help in primary healthcare.

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ORIGINAL ARTICLE

Prospective Study

Standard liver weight model in adult deceased donors with fatty liver: A prospective cohort study

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Author contributions: Li B and Chen PY contributed equally to this work; Li B collected all the clinical data, analyzed the data, drafted the manuscript and prepared the revised materials; Chen PY collected the data of donor liver weight in the operating room and assisted with data analysis; Tan YF analyzed the data, drafted the manuscript and prepared the revised materials; Huang H and Luo Y assisted with ultrasound examination and data processing; Wu ZR and Shi YJ helped with tissue staining and histological assessment; Zheng DF, He D and Jiang CH assisted with donor liver weight measurement and tissue Sampling; Jiang M participated in design of the study, data processing and data analysis; Yang JY participated in design and oversight of the study and was

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Abstract

BACKGROUND

Standard liver weight (SLW) is frequently used in deceased donor liver transplantation to avoid size mismatches with the recipient. However, some deceased donors (DDs) have fatty liver (FL). A few studies have reported that FL could impact liver size. To the best of our knowledge, there are no relevant SLW models for predicting liver size.

AIM

To demonstrate the relationship between FL and total liver weight (TLW) in detail

involved with drafting of the manuscript; all authors have read and approve the final manuscript.

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Institutional review board statement: This study was reviewed and approved by the West China Hospital of Sichuan University Institutional Review Board.

Clinical trial registration statement: This study was registered at http://www.chictr.org.cn. The registration identification number is ChiCTR2000041406.

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

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and present a related SLW formula.

METHODS

We prospectively enrolled 212 adult DDs from West China Hospital of Sichuan University from June 2019 to February 2021, recorded their basic information, such as sex, age, body height (BH) and body weight (BW), and performed abdominal ultrasound (US) and pathological biopsy (PB). The chi-square test and kappa consistency score were used to assess the consistency in terms of FL diagnosed by US relative to PB. Simple linear regression analysis was used to explore the variables related to TLW. Multiple linear regression analysis was used to formulate SLW models, and the root mean standard error and interclass correlation coefficient were used to test the fitting efficiency and accuracy of the model, respectively. Furthermore, the optimal formula was compared with previous formulas.

RESULTS

Approximately 28.8% of DDs had FL. US had a high diagnostic ability (sensitivity and specificity were 86.2% and 92.9%, respectively; kappa value was 0.70, $P < 0.001$) for livers with more than a 5% fatty change. Simple linear regression analysis showed that sex (R^2, 0.226; $P < 0.001$), BH (R^2, 0.241; $P < 0.001$), BW (R^2, 0.441; $P < 0.001$), BMI (R^2, 0.224; $P < 0.001$), BSA (R^2, 0.454; $P < 0.001$) and FL (R^2, 0.130; $P < 0.001$) significantly impacted TLW. In addition, multiple linear regression analysis showed that there was no significant difference in liver weight between the DDs with no steatosis and those with steatosis within 5%. Furthermore, in the context of hepatic steatosis, TLW increased positively (non-linear); compared with the TLW of the non-FL group, the TLW of the groups with hepatic steatosis within 5%, between 5% and 20% and more than 20% increased by 0 g, 90 g, and 340 g, respectively. A novel formula, namely, -348.6 + (110.7 x Sex [0 = Female, 1 = Male]) + 958.0 x BSA + (179.8 x FL_{US} [0 = No, 1 = Yes]), where FL was diagnosed by US, was more convenient and accurate than any other formula for predicting SLW.

CONCLUSION

FL is positively correlated with TLW. The novel formula deduced using sex, BSA and FL_{US} is the optimal formula for predicting SLW in adult DDs.

Key Words: Standard liver weight; Body surface area; Fatty liver; Sex; Deceased donors

Core Tip: This study was the first to explore the relationship between fatty liver (FL) and total liver weight (TLW) in detail using pathological biopsy based on adult deceased donors (DDs) and developed a new standard liver weight (SLW) formula. Moreover, to conveniently apply the SLW formula to the clinic, we introduced ultrasound (US). Notably, we found that FL was positively correlated with TLW and that US had a high diagnostic ability for mild to severe FL, which could increase liver weight significantly. The formula deduced using sex, BSA and FL_{US} is the optimal formula for predicting SLW in adult DDs.

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INTRODUCTION

Standard liver weight (SLW) is a key parameter in liver surgery. Its accurate evaluation is the basis for patient safety in both hepatectomy and liver transplantation

manuscript

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(LT). In hepatectomy, the underestimation of SLW may lead to residual liver failure[1,2], and in living donor liver transplantation (LDLT)/split liver transplantation (SLT), the underestimation of SLW can lead to small-for-size syndrome (SFSS)[3-5]. Since the establishment of Urata's standard liver volume (SLV) model[6], approximately 14 SLV models have been published worldwide, most of which are based on healthy people, living donors and autopsy donors from various medical centres. Deceased donor liver transplantation (DDLT) is a crucial donor liver source for alleviating the shortage of donor livers. Subsequently, SLT was established and further expanded the donor liver pool. Previous studies[7-10] have reported that SLT is not inferior to whole liver transplantation in terms of patient prognosis, which has encouraged the extensive use of SLT and necessitated an urgent demand for an SLW formula for DDLT to avoid severe mismatches, large-for-size syndrome[11,12] or SFSS. Moreover, deceased donors (DDs) and living donors (LDs) are from the general population and may have hepatic steatosis, which has a reported global incidence of 15%-30%[13,14]. To our knowledge, fatty liver (FL) may be associated with marginal grafts, as severe steatosis is a risk factor related to graft survival[15] and may affect liver size[16,17]. However, these associations have not been quantified conclusively. To the best of our knowledge, only one model[18] has been published for DDs, and it was based on a Western population and did not address FL. Therefore, this study prospectively collected adult DDs' clinical data combined with FL parameters to develop an SLW model.

MATERIALS AND METHODS

The present study prospectively enrolled consecutive deceased liver donors from West China Hospital of Sichuan University from June 2019 to February 2021 and recorded basic patient information, such as sex, age, body height (BH) and body weight (BW). This study was reviewed and approved by the West China Hospital of Sichuan University Institutional Review Board and registered at http://www.chictr.org.cn. The registration identification number is ChiCTR2000041406. All the study participants, or their legal guardians, provided informed written consent prior to study enrollment, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethics committee. No executed prisoners were included in the study. A total of 212 DDs were enrolled, and brain death was confirmed in all of them before organ procurement. Advanced life support was maintained in an intensive care unit (ICU); moreover, abdominal ultrasound (US) examinations, liver function tests and kidney function tests were completed for each donor. Pathological biopsy (PB) was performed for all enrolled donor livers after they were obtained.

US examination

A US examination was carried out for all DDs before organ procurement. Scanning and diagnosis were conducted by 2 experienced (> 5 years) US doctors who were blinded to the final PB diagnosis. The examinations were performed by using a MultiWave ultrasound system (Aixplorer, France) equipped with an SC6–1 (1–6 MHz) transducer. FL was identified as a diffuse increase in fine echoes in the liver parenchyma. Representative images[19] are presented in Figure 1.

Donor liver weight measurement, tissue sampling and histological assessment

Donor livers were procured and trimmed in the operating room and were then weighed with a precision electronic balance (unit: kg, accurate to 0.001 kg, Figure 2) on a back table.

A single tissue wedge of approximately 1.0 cm × 1.0 cm × 1.0 cm was excised from the left lateral lobe surface of the donor liver, fixed in formalin and embedded in paraffin. Each donor liver was stained with haematoxylin and eosin (HE) and Masson's trichrome. The histological degree of liver pathology, including hepatic steatosis, ballooning of hepatocytes, lobular inflammation, necrosis, and fibrosis, was evaluated by two expert liver pathologists blinded to any other clinical information and laboratory data. The extent of hepatic steatosis was assessed by the percentage of hepatocytes containing large- and medium-sized intracytoplasmic lipid droplets (but not foamy microvesicles). The definition of ballooning of hepatocytes and lobular inflammation was as described by Kleiner *et al*[20] and Bedossa *et al*[21]. The definition of necrosis is described in Table 1. Fibrosis was scored according to the standard grading (inflammation) and staging (fibrosis) method based on the modified Scheuer

Table 1 Characteristics of the deceased donors

Characteristic	Total, *n* = 212
Sex, male, *n* (%)	167 (78.8)
Age, median (range), yr	49 (18–68)
BH, median (range), cm	168 (150–185)
BW, median (range), kg	65 (45–90)
BMI, median (range), kg/m^2	23.35 (15.57–30.48)
BSA, median (range), m^2	1.73 (1.37–2.10)
TLW, median (range), g	1400 (830–2100)
Cause of death, *n* (%)	
Trauma	106 (50.0)
Cerebrovascular	97 (45.8)
Other	9 (4.2)
Degree of fatty change, median (range)	0 (0–40%)
0, *n* (%)	151 (71.2)
> 0, < 5%, *n* (%)	32 (15.1)
5%–33%, *n* (%)	22 (10.4)
> 33%, *n* (%)	7 (3.3)
Ballooning of hepatocytes	
None	24 (11.1)
Ballooned hepatocyte with normal size	116 (54.9)
Enlarged ballooned hepatocyte	72 (34.0)
Lobular inflammation	
None	66 (30.9)
< 2 foci per lobule	131 (61.7)
> 2 foci per lobule	15 (7.4)
Necrosis	
None	200 (94.4)
Focal or unicellular necrosis	8 (3.7)
More extensive necrosis and above	4 (1.9)
Stage of fibrosis[1]	
0	72 (33.8)
1	88 (41.6)
2	47 (22.1)
3	4 (1.9)
4	1 (0.6)

[1]According to the modified Scheuer system[22]. BH: Body height; BW: Body weight; BMI: Body mass index; BSA: Body surface area; TLW: Total liver weight.

system[22].

Estimating SLW using previous formulas

According to previous studies at our centre[23] and other centres[24-26], the density of the liver was determined to be 1 g/cm^3; that is, the weight and volume of the donor liver were equal. For comparison, we calculated the estimated SLW according to

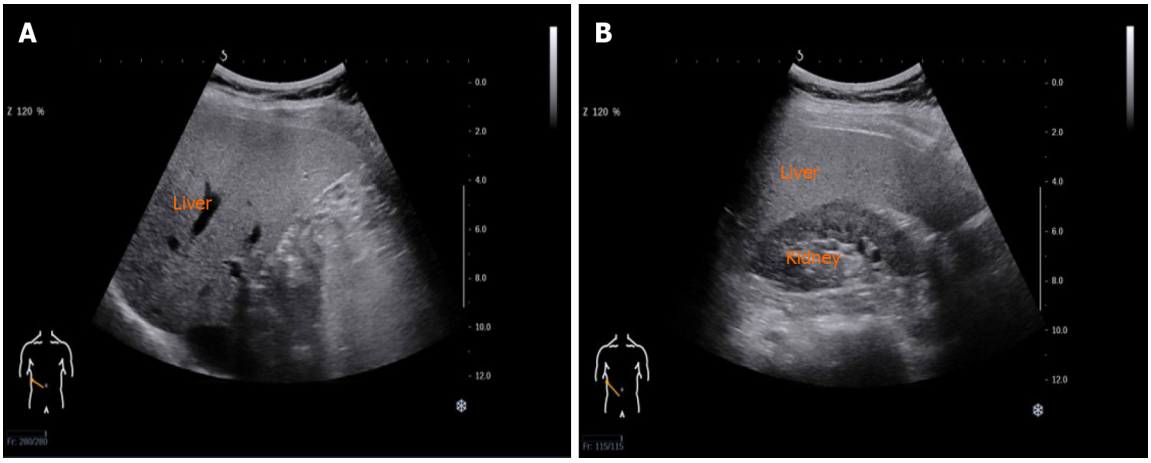

Figure 1 Diagram of fatty liver diagnosed by ultrasound from the view of the liver and kidney. A: Diffuse increase in fine echoes in liver parenchyma with normal visualization of intrahepatic vessel borders; B: Diffuse increase in fine echoes in liver parenchyma. There was an increase in echogenicity of the liver compared with the echogenicity of the renal cortex.

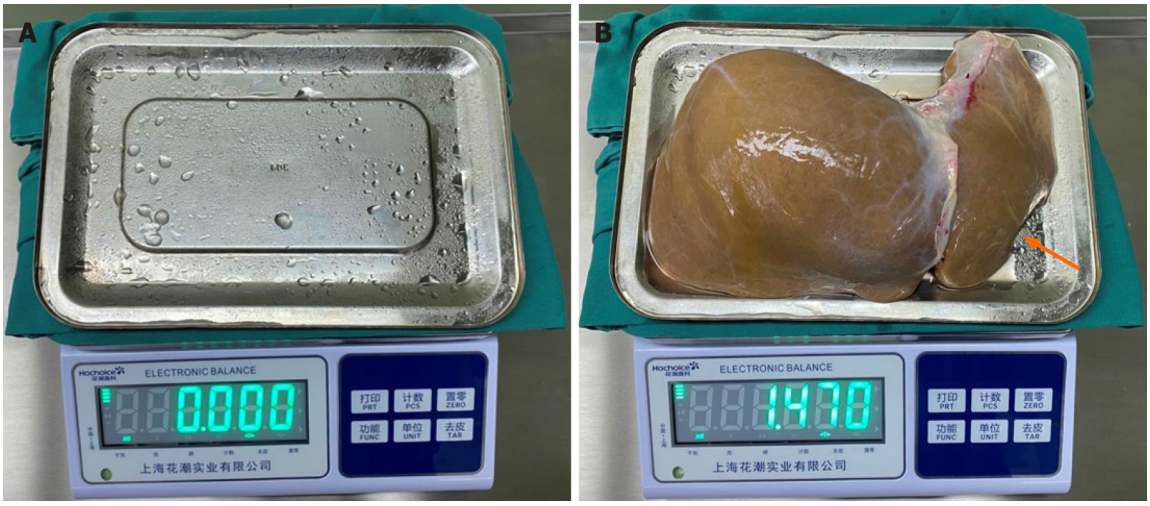

Figure 2 Actual liver weight measurement by electronic balance. A: Zero correction of electronic balance; B: Donor liver weighing. The arrow indicates that a single tissue wedge of approximately 1.0 cm × 1.0 cm × 1.0 cm was excised from the left lateral lobe surface of the donor liver.

previous formulas for adults[6-19]. Body mass index (BMI) = BW/BH^2 and body surface area (BSA) = $BW^{0.425}$ x $BH^{0.725}$ x 0.007184 using the Dubois formula[27] were also calculated.

Statistical analysis

In this study, simple linear regression analysis was used to explore the variables related to TLW. Multiple linear regression analysis was used to formulate the SLW. As BH, BW, BMI and BSA are collinear variables, each was applied in a different prediction model. The root mean standard error (RMSE) and interclass correlation coefficient (ICC) were used to test the fitting efficiency and accuracy of the model, respectively. The chi-square test and kappa consistency score were used to assess the consistency in terms of FL diagnosed by US relative to PB. Continuous variables were analysed by a paired-samples *t* test. Two-tailed statistical analysis was used, and *P* values less than 0.05 were considered to be statistically significant. SPSS, version 25.0 (IBM, Armonk, NY, United States) was used for all statistical analyses. GraphPad Prism 7.0 (GraphPad Software, Inc.) was used for drawing.

RESULTS

Baseline data

This study included 167 males (78.8%). The median age was 49 years, ranging from 18 to 68 years. The median BH, BW, BMI, BSA and TLW were 1.68 m, 65 kg, 23.35 kg/m^2, 1.73 m^2 and 1400 g, respectively. The main causes of death of the DDs were trauma (50%), cerebrovasculature (45.8%), and other (4.2%), which included brain tumours and hypoxic-ischaemic encephalopathy. There were 151 DDs (71.2%) with no steatosis, 32 (15.1%) with steatosis within 5%, 22 (10.4%) with steatosis between 5% and 33%, and 7 (3.3%) with steatosis greater than 33%. Moreover, hepatocyte ballooning was observed in 88.9% of DDs. Lobular inflammation was observed in approximately 69.1% of DDs. Necrosis (focal or unicellular necrosis, in 3.7% of DDs samples, and more extensive necrosis, in 1.9% of DDs samples) was observed in only a few DDs liver tissue samples. Stage 0–2 Liver fibrosis was observed in approximately 97.5% of DDs (Table 1).

Impact factors related to the TLW of deceased donors

Simple linear regression analysis showed that sex, BH, BW, BMI, BSA and FL significantly impacted TLW ($P < 0.001$) (Table 2). BSA was the most influential factor related to liver size [R^2, 0.454; 95% confidence interval (CI): 1024.56–1383.79]. Multiple linear regression analysis showed that there was no significant difference in TLW between no steatosis and steatosis within 5% ($P = 0.147$, Figure 3A). Furthermore, in the context of hepatic steatosis, TLW increased positively (non-linear); compared with the TLW of the non-FL group, the TLW of the groups with hepatic steatosis within 5%, between 5% and 20% and more than 20% increased by 0 g, 90 g, and 340 g, respectively (Figure 3B).

Consistency test for FL diagnosis between US and PB

This study investigated 61 hepatic steatosis cases, which accounted for 28.8% of all cases, and moderate and severe steatosis cases, which accounted for 3.3%. The cases of hepatic steatosis and non-hepatic steatosis diagnosed by US were 38 and 174, respectively. The sensitivity and specificity of US were 55.7% and 97.4%, respectively, and the kappa value was 0.598 ($P < 0.001$). That is, its diagnostic consistency was good (Supplementary Table 1). Furthermore, when setting 5% as the cut-off value for diagnosing FL by PB, there were 174 cases within a 5% fatty change and 38 cases with more than a 5% fatty change diagnosed by US, with a sensitivity and specificity of 86.2% and 92.9%, respectively, and a kappa value of 0.70 ($P < 0.001$). Therefore, the diagnostic consistency between US and PB was high (Table 3).

Current formulas for estimating SLW

The SLW models were separately formulated based on four collinear variables, namely, BH, BW, BMI and BSA. Subsequently, three prediction model groups were established, two of which were used to assess the presence of FL based on US or PB; the third group did not include FL as an indicator. The present study showed that the SLW models based on BSA, FL and sex had the best fitness, and the adjusted R^2 and RMSE for PB and US were 0.546 and 169.985 and 0.546 and 169.913, respectively. The fitting efficiency of these two models was almost equal and better than that of the traditional method (adjusted R^2, 0.485; RMSE, 181.095) (Table 4).

Comparison between the current formula and previous formulas

Previously reported formulas were used to assess our DDs cohort, and the results showed that the fitting efficiency and accuracy of the SLW model introducing FL diagnosed by US were 168.3 (RMSE) and 0.71 (ICC), with a non-significant difference ($P = 1.00$) between the SLW and TLW of 1.5 g. The RMSE and ICC of Yu *et al*[25]'s and Lin *et al*[28]'s models were 187.5 and 0.61 and 188.0 and 0.63, respectively. There were no significant differences between the SLW and actual TLW for these two formulas, but those of the remaining formulas were significantly different (Table 5)[6,18,25-37].

DISCUSSION

The shortage of donor livers is a problem worldwide and has become a major obstacle hindering the development of LT. To date, experts in the LT field have explored expanding the donor liver pool, including *via* SLT, marginal donor LT, domino LT and

Table 2 Factors related to the total liver weight of the deceased donors

Factor	R^2	*P* value	95%CI
Sex	0.226	< 0.001	220.89–369.68
BH	0.241	< 0.001	13.92–22.78
BW	0.441	< 0.001	15.25–20.77
BSA	0.454	< 0.001	1024.56–1383.79
BMI	0.224	< 0.001	32.28–54.18
Degree of fatty change (< 5%, 5%–20%, > 20%)	0.130	< 0.001	116.89–244.17
Hepatic steatosis[1]	0.125	< 0.001	149.67–318.33

[1]Diagnosed by ultrasound. BH: Body height; BW: Body weight; BSA: Body surface area; BMI: Body mass index.

Table 3 Results for livers with more than 5% fatty change diagnosed by ultrasound and pathological biopsy in the deceased donors

Ultrasound	Pathological biopsy		Total
	+	-	
+	25	13	38
-	4	170	174
Total	29	183	212

According to the table above, livers with a fatty change of more than 5% were diagnosed by ultrasound, and the sensitivity and specificity were 86.2% and 92.9%, respectively. The chi-square test showed that the kappa value was 0.70, $P < 0.001$.

so on. These schemes have successfully and significantly expanded the donor liver pool, and SLT has become one of the most valuable means of promotion. Graft weight (GW) plays a key role in recipients, especially in DDLT and LDLT. Therefore, it is necessary to evaluate the donor liver size in LT.

DDs are patients with brain death caused by non-liver diseases. This study illustrated that 95.8% of DDs died from trauma or cardiovascular and cerebrovascular accidents. Biopsies showed that many donor livers had hepatocyte oedema and lobular inflammation, which can be explained by the cause of death. Trauma and cardiovascular and cerebrovascular accidents can cause instability of the circulatory system, leading to long-term ICU stays and the requirement for resuscitative therapy, which may cause unstable organ perfusion (hypoperfusion or hyperperfusion) and reperfusion injury. In addition, the use of a large number of vasoactive drugs may aggravate organ microcirculation disorder. Thus, the graft may have acute injury, such as lobular inflammation, hepatocyte oedema and even necrosis. The present study found that 28.8% of DDs had hepatic steatosis and that 2.5% had stage 3–4 Liver fibrosis. Unlike DDs, LDs screened from healthy populations rarely have FL or other acute liver injuries. In addition, it was unclear whether there was a difference in the SLW between DDs and LDs. To the best of our knowledge, there have been few relevant reports. Therefore, we explored the SLW model based on DDs data derived from West China Hospital.

Simple linear regression analysis showed that liver size was correlated with sex. The liver size of males was larger than that of females, which was in line with previous studies[30,33]. We speculated that this might be related to the fact that the body size of men is generally larger than that of women and that men have a larger skeletal muscle system and higher daily consumption and metabolic requirements. Therefore, a larger liver mass is needed to meet physiological needs[38,39]. In addition, the present study found that BH, BW, BMI and BSA were closely related to liver size, which was similar to previous studies[6,25,31,40]. Indeed, multiple linear regression analysis revealed that the above four variables were collinear. From the perspective of morphology, liver size and physical indicators are supposed to be positively correlated. Moreover, in terms of energy requirements, to meet metabolic needs, a larger body size needs more organ support. Furthermore, the current study found that BSA was the most influential factor impacting TLW, which was consistent with previous studies[6,29,

Table 4 Results of multiple linear regression analysis performed to predict the total liver weight using each of the body anthropometric measures divided into groups of the traditional method and two new methods, which introduce the parameter of fatty liver diagnosed by ultrasound and pathological biopsy

Groups	Formulas	Adjusted R^2	RMSE
Traditional method			
BH	- 809.4 + 167.3 x Sex + 12.6 x BH	0.29	212.0
BW	322.1 + 147.0 x Sex + 15.2 x BW	0.49	181.1
BSA	- 466.9 + 99.0 x Sex + 1051.0 x BSA	0.48	182.8
BMI	329.2 + 264.5 x Sex + 37.8 x BMI	0.39	196.5
Ultrasound method			
BH	- 1011.9 + 149.7 x Sex + 13.6 x BH + 240.7 x FL_{US}	0.43	191.1
BW	392.7 + 158.3 x Sex + 13.5 x BW + 158.6 x FL_{US}	0.54	171.4
BSA	- 348.6 + 110.7 x Sex + 958.0 x BSA + 179.8 x FL_{US}	0.55	169.9
BMI	453.7 + 264.5 x Sex + 31.2 x BMI + 162.9 x FL_{US}	0.45	187.5
Pathological biopsy method (< 5%, 5%–20%, > 20%)			
BH	- 803.7 + 178.5 x sex + 12.3 x BH + FL_{PB} (0 = 0, 1 = 163.5, 2 = 393.0)	0.43	190.0
BW	414.5 + 172.6 x sex + 13.1 x BW + FL_{PB} (0 = 0, 1 = 79.8, 2 = 280.7)	0.54	170.8
BSA	- 288.8 + 129.5 x sex + 919.6 x BSA + FL_{PB} (0 = 0, 1 = 93.9, 2 = 304.5)	0.55	170.0
BMI	478.1 + 276.5 x Sex + 30.0 x BMI + FL_{PB} (0 = 0, 1 = 105.3, 2 = 299.1)	0.46	185.4

Sex and FL_{US} are binary variables; FL_{PB} is a dummy variable. Sex: 0 = Female, 1 = Male; FL_{US}: 0 = No, 1 = Yes; FL_{PB}: 0 < 5%, 1 = 5%–20%, 2 > 20%. BH: Body height; BW: Body weight; BSA: Body surface area; BMI: Body mass index; FL_{US}: Fatty liver diagnosed by ultrasound; FL_{PB}: Fatty liver diagnosed by pathological biopsy; RMSE: Root mean standard error.

31]. BSA is a widely used parameter in physiology and clinical medicine for normalizing biological function with respect to variations in body size and conformation. Thus, we believe that the liver size required to meet the metabolic demands of the individual may correlate more closely with BSA than with any other parameter. Additionally, previous studies[30,34] reported that age was associated with TLW; however, similar to Poovathumkadavil's study[35], we failed to identify an association between age and TLW. Several previous studies[31,40] reported that the partial regression coefficient of age was very small, and the authors considered the effect of this variable in adults to be negligible. Therefore, our negative result may be explained by the age distribution of patients in our study and the sample size, and further studies with larger sample sizes are needed to confirm the relation between age and TLW.

Interestingly, this study found that more than a quarter of DDs from the general population had hepatic steatosis, which was similar to Zhou *et al*[41]'s report (29.2%). To our knowledge, an increasing number of individuals, especially those who are obese, suffer from FL worldwide[42,43]. Furthermore, the present study also found that 10.4% and 3.3% of livers had mild and moderate steatosis, respectively, while no liver was detected to have severe steatosis. Several studies have confirmed that mild steatosis grafts (< 33%) can be used safely in LT. However, the eligibility of livers with moderate steatosis is controversial, while livers with severe steatosis are generally discarded because of the increased probability of primary non-function[15,44,45]. Importantly, in the current study, simple linear regression analysis demonstrated that FL was correlated with TLW. Moreover, multivariate analysis showed that steatosis significantly affected TLW, and the degree of steatosis was positively correlated with liver size, which was consistent with previous studies[16,46,47]. Multiple linear regression analysis showed that compared with non-FLs, the presence of hepatic steatosis within 5%, 5%–20% and over 20% resulted in an increase in liver weight by 0 g, 93.9 g, and 304.5 g, respectively. In LT, we generally evaluate the feasibility of SLT

Table 5 Differences between the estimated and actual liver weights calculated using previous formulas in our deceased donor cohort.

Ref.	Formula	Difference[1] (g)	RMSE	ICC	*P* value[2]
Autopsy					
DeLand *et al*[29]	1020 × BSA - 220	135.5 (-366–632)	221.2	0.52	< 0.01
Heinemann *et al*[26]	1072.8 × BSA - 345.7	95 (-421–556)	202.5	0.56	< 0.01
Yu *et al*[25]	$21.585 \times BW^{0.732} \times BH^{0.225}$	34.5 (-490–576)	187.5	0.61	0.102
Choukèr *et al*[30]	[16–50 yr] 452 + 16.34 x BW + 11.85 × age - 166 × sex (1 = female, 0 = male) 51–70 yr] 1390 + 15.94 × BW - 12.86 × age	435 (-301–1000)	484.0	0.24	< 0.01
General population/living donor					
Urata[6]	706.2 × BSA + 2.4	-185 (-713–337)	278.1	0.32	< 0.01
Lin *et al*[28]	13 × BH + 12 × BW - 1530	11.5 (-546–445)	188.0	0.63	0.472
Vauthey *et al*[31][3]	1267.28 × BSA - 794.41	-15 (-544–421)	188.1	0.64	< 0.01
Hashimoto *et al*[32]	961.3 × BSA - 404.8	-161 (-668–317)	253.4	0.42	< 0.01
Chan *et al*[33]	218 + BW × 12.3 + sex × 51 (0 = female, 1 = male)	-356.5 (-859–175)	411.1	0.21	< 0.01
Yuan *et al*[34]	949.7 × BSA - 247.4–48.3 x age factor (1, < 40; 2, 41–60; 3, > 60)	-106 (-646–359)	228.0	0.48	< 0.01
Fu-Gui *et al*[23]	11.508 × BW + 334.024	-319 (-845–241)	393.6	0.19	< 0.01
Poovathumkadavil *et al*[35]	12.26 × BW + 555.65	-57 (-572–510)	207.5	0.47	< 0.01
Um *et al*[36]	893.485 x BSA − 439.169	-312.5 (-816–173)	372.8	0.24	< 0.01
Cadaveric population					
Yoshizumi *et al*[18][3]	772 × BSA	-79 (-602–416)	214.6	0.45	< 0.01
Current	- 348.6 + 110.7 x Sex (0 = Female, 1 = Male) + 958.0 x BSA + 179.8 x FL_{US} (0 = No, 1 = Yes)	1.5 (-477.0–450.0)	168.3	0.71	1

[1]Difference between estimated and actual liver weight using previous formulas.
[2]Paired-samples *t* test.
[3]Mosteller's formula[37] was adopted for BSA, and the remaining formulas used the Dubois formula[27].
BH: Body height; BW: Body weight; BSA: Body surface area; BMI: Body mass index; FL_{US}: Fatty liver diagnosed by ultrasound; ICC: Interclass correlation coefficient; RMSE: Root mean standard error.

based on the criteria of GW/SLW (30%–40%) or GW/BW (0.8%)[11]. Thus, for FL, the GW required for recipients would be underestimated if calculated according to the traditional SLV method, leading to an increased risk of SFSS. Therefore, the current study introduced the FL variable for the first time to develop an SLW model. To diagnose FL before organ procurement, US was performed for all DDs. Notably, for a diagnosis of mild steatosis and greater (≥ 5%), the sensitivity and specificity of US were 86.2% and 92.9%, respectively, and the ICC was 0.70 ($P < 0.001$). That is, US had a higher diagnostic consistency with PB. In addition, this study revealed that the size of livers with a fatty change less than 5% was not different from that of livers without fatty change but was different from that of livers with a fatty change of 5% or greater. The gap of liver size between these two hepatic steatosis categories was significant (180 g, $P < 0.001$), which laid a solid theoretical foundation to apply US in the diagnosis of FL and develop the SLW model, highlighting its clinical practical value.

In this study, the deduced best fit formula based on US had equivalence with that based on PB and was better than the best fit traditional model. Furthermore, the present study showed that the formulas of Deland *et al*[29], Heinemann *et al*[26], and Choukèr *et al*[30] overestimated liver size, while the formulas of Urata *et al*[6], Vauthey *et al*[31], Yoshizumi *et al*[18], Hashimoto *et al*[32], Chan *et al*[33], Yuan *et al*[34], Fu Gui *et al*[23], Poovathumkadavil *et al*[35], and Um *et al*[36] underestimated liver size. On the other hand, there was no significant difference between the actual liver weight and the predicted liver weight calculated by Yu *et al*[25]'s and Lin *et al*[28]'s formulas. This was speculated to be related to the characteristics of the study samples. Deland *et al*[29]'s, Heinemann *et al*[26]'s and Choukèr *et al*[30]'s cohorts were autopsy samples. To

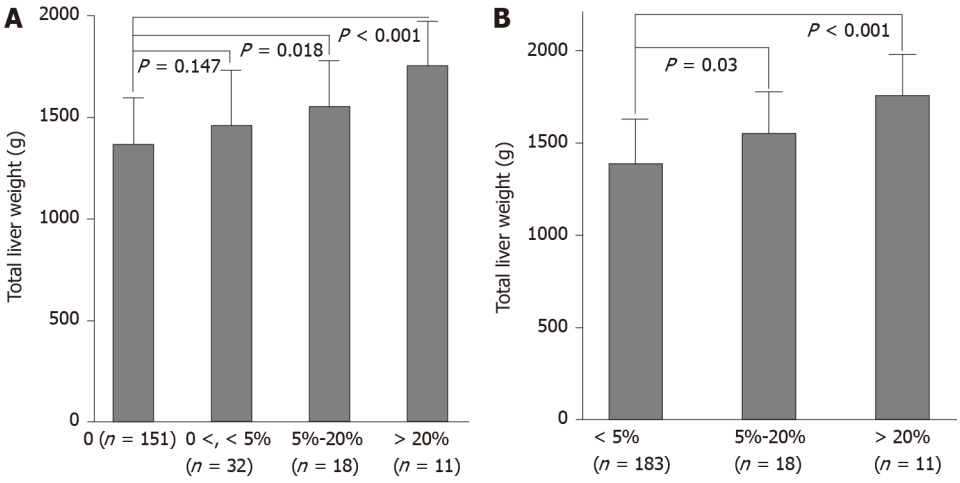

Figure 3 Total liver weight comparison of different groups according to the degree of fatty change of donor livers. A: Groups according to the degree of fatty change of 0, (0 <, < 5%), (5%–20%) and > 20%; B: Groups according to the degree of fatty change of < 5%, (5%–20%) and > 20%. Multiple linear regression analysis including parameters of sex, BSA, and FL_{PB}, which were dummy variables divided into groups according to the degree of fatty change in donor livers, was used. FL_{PB}, fatty liver diagnosed by pathological biopsy.

our knowledge, data from autopsy studies[29] includes the weight of the gallbladder, the attached ligaments, and the hepatic vena cava. In addition, various causes of death, *i.e.*, cardiac failure and traffic accidents, might increase liver weight through mechanisms associated with shock-related hepatic congestion. On the other hand, due to long-term immersion in the fixed solution, the weight of the specimen may exceed the actual size *in vivo*. However, the autopsy study of Yu *et al*[25] was not consistent with the other three autopsy studies but was similar to our study, which may be explained by racial differences. Additionally, the cohorts of Vauthey *et al*[31], Hashimoto *et al*[32], Chan *et al*[33], Yuan *et al*[34], Fu Gui *et al*[23], Poovathumkadavil *et al*[35], and Um *et al*[36] were based on healthy populations without liver disease. However, Lin *et al*[28]'s study cohort comprised 44 (57.1%) patients with chronic liver disease (alcoholic hepatitis, 9; hepatitis B, 24; and hepatitis C, 11), which may explain the difference from other studies based on the general population. Notably, the difference was significant between actual liver weight and estimated liver weight using the formula of Yoshizumi *et al*[18], which was the only previous study based on a cadaveric population. Their study included DDs of several races, most of which were Western, and subjects under 18 years were enrolled. These confounding factors may explain the difference. Therefore, for different study populations, the model for predicting liver size is supposed to be different, which highlights the need for this study for adult DDs. In addition, this study shows the practicability and rationality of the current SLW model in DDLT. Theoretically, it suggests that the current formula is the most suitable for recipients assigned with FL in SLT, and use of this formula is anticipated to reduce the risk of SFSS.

However, the sample size of this study was relatively small, especially in regard to cases of moderate to severe hepatic steatosis. Therefore, studies with larger sample sizes are warranted to optimize the SLW model. Additionally, the extrapolation and clinical practicability of the current SLW model need to be further verified.

CONCLUSION

In conclusion, this study was the first to demonstrate the positive correlation between the degree of hepatic steatosis and liver size based on pathological findings. Furthermore, this study creatively proposed and verified the equivalent value of FL diagnosed by US instead of that diagnosed by PB in terms of the FL variable in the SLW model as follows: SLW (g)= -348.6 + [110.7 x Sex (0 = Female, 1 = Male)] + 958.0 x BSA + [179.8 x FL_{US} (0 = No, 1 = Yes)]. This formula can be used to estimate the liver weight before liver procurement. Additionally, our formula lays a theoretical and practical basis for the further application of donor livers with fatty changes in SLT.

ARTICLE HIGHLIGHTS

Research background

Standard liver weight (SLW) is frequently used in liver transplantation, especially for living donor liver transplantation/split liver transplantation (SLT). However, some deceased donors (DDs) have fatty liver (FL). There have been a few studies to report that FL could impact liver size. This study was to develop a new formula including FL to predict liver size.

Research motivation

To explore SLW model in adult DDs with FL and help transplant doctors make allocation decisions, especially for recipients assigned with FL in SLT to reduce the risk of small-for-size syndrome.

Research objectives

To explore the liver pathology of DDs, such as hepatic steatosis, and diagnostic ability of ultrasound for FL, as well as the relationship between FL and total liver weight. Furthermore, to develop an SLW formula, combined with FL parameter, used to predict graft weight required for recipients in SLT.

Research methods

This study prospectively enrolled consecutive DDs from West China Hospital of Sichuan University from June 2019 to February 2021 and recorded basic patient information, and abdominal ultrasound (US) examination and pathological biopsy (PB) were performed for them. Furthermore, the chi-square test and kappa consistency score were used to assess the consistency in terms of FL diagnosed by US relative to PB. Simple linear regression analysis was used to explore the variables related to TLW. Multiple linear regression analysis was used to formulate SLW models.

Research results

More than a quarter of DDs had hepatic steatosis, and US had a high diagnostic ability for mild to severe FL. Furthermore, this study found that FL was positively correlated with liver size and deduced an optimal SLW formula in adult DDs with FL. However, the extrapolation and clinical practicability of the current SLW model need to be further verified in the future.

Research conclusions

FL is positively correlated with liver size. Our novel formula deduced using sex, BSA and FL_{US} is the optimal formula for predicting SLW in adult DDs with FL.

Research perspectives

To verify the extrapolation of the current SLW model using multicentre data and its clinical practicability in SLT.

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SYSTEMATIC REVIEWS

Microbiota shaping — the effects of probiotics, prebiotics, and fecal microbiota transplant on cognitive functions: A systematic review

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Abstract

BACKGROUND

Dementia is a chronic progressive neurological disease affecting millions of people worldwide, and represents a relevant economic burden for healthcare systems. Although its pathogenesis is still unknown, recent findings have reported that a dysregulated gut-brain axis communication, a fundamental relationship mediated by several host and microbial molecules, is associated with cognitive disorders. In addition, gut microbiota manipulation reduces neuroinflammation, improving cognitive function by restoring the functional gut-brain axis.

AIM

To better define the effects of probiotics, prebiotics, synbiotics, and fecal microbiota transplant (FMT) on cognitive function.

METHODS

We performed a literature search of human randomized clinical trials to examine the effects of the administration of probiotics, prebiotics, synbiotics, or FMT on cognition outcomes in healthy or sick people of every age, sex, and nationality. We systematically searched Embase, Medline/PubMed, Cochrane Library, central and clinicaltrials.gov databases with a combination of comprehensive terms related to cognition and gut microbiota manipulation. Then we carefully reviewed and synthesized the data by type of study design and setting, characteristics of the studied population, kind of intervention (strain type or mixture type, dosage, and frequency of administration), control treatment, inclusion and exclusion criteria, follow-up duration, and cognitive or memory outcomes.

RESULTS

After examining the titles and abstracts, the initial literature screening identified

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995 articles, but we added 23 papers in our systematic review. The analyses of these selected studies highlighted that both probiotic supplementation and FMT improved cognitive function regardless of the type and posology of administration and the adopted cognitive tests and questionnaires. We found that most of the studies conducted in healthy people showed a significant positive effect of the intervention on at least one of the performed cognitive tests. Regarding unhealthy subjects, while FMT and especially probiotic administration had multiple beneficial effects on different cognitive functions, supplementation with prebiotics did not provide any cognitive improvement.

CONCLUSION
Probiotic supplementation and FMT may represent a promising strategy to restore gut eubiosis and enhance the cognitive functions of healthy people and patients with neurological disorders.

Key Words: Dementia; Cognitive disorders; Gut microbiota; Probiotics; Prebiotics; Fecal microbiota transplant

Core Tip: Dementia and cognitive impairment are age-related conditions that are on the rise worldwide. Recent studies have demonstrated the existence of a gut-brain axis and that the manipulation of gut microbiota composition can exert positive effects on cognition. The administration of probiotics, prebiotics, and fecal microbiota transplant may represent a good strategy to counteract gut dysbiosis and ameliorate cognitive dysfunction by reducing neuroinflammation and brain damage.

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INTRODUCTION

Global population ageing, defined as the increasing proportion of older people around the globe, represents a deep shift in society and a considerable challenge for the sustainability of healthcare systems due to the rise of geriatric illnesses[1,2]. Currently, the prevalence of cognition impairment, particularly dementia, is estimated worldwide in 50 million people with an economic burden of 818 billion dollars in 2016 and a forecast of about 115 million people by 2050[3,4].

Dementia is an acquired, gradual, and progressive disorder involving multiple adverse neurocognitive changes that can affect learning processes, memory, executive function, language, complex attention, mood, perceptual-motor function or social cognition. Moreover, although its detailed pathological mechanism is still not well understood, dementia often occurs in association with advanced age or the presence of contributing causes, usually Alzheimer's disease (AD), Parkinson's disease (PD), or cerebrovascular pathology[5-7]. Unfortunately, current therapies are only symptomatic, and notably, no treatment stops the disease progression[8].

Recent studies have shown that that the gut microbiota, with more than 100 trillion microorganisms carrying three times of human genes, plays a pivotal role in human health; manipulation of the intestinal microbiota can modify the release of neuroactive metabolites, which affect brain health[9,10]. This role can be further explained by the documented existence of the gut-brain axis, a complex bidirectional system in which communication occurs through three parallel and interplaying pathways that involve nervous, endocrine, and immune signals[11]. Therefore, different preclinical and observational studies have demonstrated that the gut dysbiosis is responsible for increased intestinal permeability, which correlates with both neuroinflammation and a decline of cognitive abilities[12-15].

Dietary interventions (in nutritional supplements or specific diets) have often been applied in clinical practice to restore intestinal eubiosis and prevent and treat cognitive disorders. For example, a Mediterranean diet and/or a healthy diet based on fruits, vegetables, and fish seems to stabilize or slow cognitive decline[16].

Nevertheless, the most promising strategy to counteract gut dysbiosis and to maintain cognitive function seems represented by the administration of probiotics, prebiotics, and fecal microbiota transplant (FMT).

Interestingly, administration in animal models of an adequate posology of multistrain probiotics reduces both Firmicutes/Bacteroidetes ratio and intestinal permeability, slowing cognitive decline and reducing neuroinflammation[17,18]. Moreover, using specific prebiotics seems to ameliorate cognitive performance with a direct effect on gut microbiota[19].

Moreover, even FMT has demonstrated remarkable efficacy in healthy subjects and people affected by various diseases caused by gut microbiota perturbation, particularly *Clostridium difficile* infection. It could represent a promising therapy for cognitive impairment improvement because of its capability to re-establish a healthy gut microbial community[20,21].

Therefore, since the evidence derived from human randomized clinical trials (RCTs) is currently limited, this systematic review identified the available RCTs and better defined the effects of probiotics, prebiotics, synbiotics, and FMT on cognitive functions.

MATERIALS AND METHODS

Literature search

Our study followed the PRISMA statement guidelines. A computerized search of the articles published until 24 October 2019 was conducted in Embase, Medline/PubMed, Cochrane Library, central and clinicaltrials.gov databases and other individual journal sources, using the following search string: (memory OR cognition OR dementia) AND (lactobacillus OR bifidobacteria OR streptococcus OR enterococcus OR probiotic OR prebiotic OR symbiotic OR fecal, transplantation). In the PubMed database, we activated the filter "Humans"; in Embase, we selected the filter "Research articles"; in Cochrane Library, we activated the filter "Trials"; and in clinicaltrials.gov, we selected the filter "recruitment: terminated or completed." The search did not apply filters for language, country, duration of follow-up, and participants' characteristics (age and sex).

Study selection

Two authors independently reviewed the titles and abstracts of the collected articles, applying predefined inclusion/exclusion criteria. The inclusion criteria were as follows: RCTs; availability of full text; patients regardless of age, nationality, sex, and health status; comparison between oral intake of probiotics, prebiotics, or symbiotic and control treatment or placebo; and outcome as cognitive or memory evaluation. The adopted exclusion criteria were as follows: Studies with fewer than 10 participants; reviews, articles, and case reports; or studies with incomplete outcomes.

Data extraction

The same two authors performed analyses of the full text and data extraction with the intervention of a third author in case of poor agreement or discrepancies. Each reviewer independently recorded the data in a predefined data extraction form. The following data, if reported, were obtained from each selected trial: First author name, year of publication, study design, setting (institution, city, and country), characteristics of the studied population (mainly age and health status), number of total participants and their gender, number of subjects in both treatment and control groups, characteristics of the intervention (strain type or mixture type, dosage and frequency of administration), control treatment, inclusion and exclusion criteria, follow-up duration, cognitive or memory outcomes and compliance data.

Outcome assessment

For each selected study, cognitive functions were assessed through specific tests which evaluated the eight main cognitive skills: sustained attention, speed of information processing, cognitive flexibility and control, multiple simultaneous attention, working memory (short-term memory), category formation, pattern recognition, and response

inhibition[22]. A detailed description of all cognitive tests performed in the selected papers for this systematic review is annexed in Supplementary material.

RESULTS

Study selection

The initial literature screening identified 995 papers. Eight studies were excluded for duplication and another 964 papers were removed after the title and abstract screening because they did not respect inclusion criteria. The selection process, in accordance with the PRISMA statement 2009, is illustrated in Figure 1.

Characteristics of the included studies

An overview of the 23 studies included in this systematic review is reported in Table 1. All 23 included papers were RCTs published from 2007 to 2019[23-45]. The total number of participants was 1285 (491 males and 650 females); unfortunately, both articles published by Tamtaji *et al*[44,45] did not report the gender of the participants. Regarding the age of the enrolled subjects, one study was conducted in healthy scholars (7-9 years)[31], four studies enrolled young adults (19-30 years)[25,36,40,43], and most of the studies involved adults or older people (48-95 years)[23,24,26-30,32-35,37-39,40,42,44,45]. Most studies (four) were performed in Iran[22,24,44,45]; three in the United States[26-28] and Japan[34,37,39]; two in the United Kingdom[29,31], South Korea[33,35], and Spain[30,41]; and one in Austria[25], Italy[32], Ireland[36], Malaysia [38], Poland[42], Wales[43] and the Netherlands[40].

Concerning the patients' health state, most studies enrolled healthy people[25,29,31,33-36,40,43], whereas three studies involved patients with AD[23,24,44] or cirrhotic subjects with recurrent encephalopathy[26-28]. The other studies were focused on stressed adults[38], patients with PD[45], human immunodeficiency virus (HIV)-1-infected individuals[32], subjects affected by fibromyalgia syndrome[41], people with major depression[42], elderly with frailty syndrome[30], and adults with forgetfulness [39] and mild cognitive impairment[37].

In the trials, subjects were administered probiotics[22-25,26,29,32-42,44,45], prebiotics[30,31,43], or FMT[27,28] and its duration lasted a maximum of 24 wk[32] and a minimum of 4 h[43]; however, the trials continued for 12 wk for most of the studies[23,24,31,33-35,37,38,44,45]. No studies have reported the administration of synbiotics. In the studies examining the effects of probiotics, a total of 21 different bacterial species were administered (alone or in combination) in a dosage ranging from 1×10^9 CFU/mL to 2.5×10^{10} CFU/mL; the most represented species were *Lactobacillus plantarum*, *L. acidophilus*, and *Bifidobacterium bifidum*. On the other side, the administered prebiotics was composed of inulin or galacto-oligosaccharides (GOS), in a dosage that ranged from 5 g/d to 7.5 g/d. In FMT studies, subjects were administered a capsule containing 550 μL stool and buffer solution or enema infusion of 90 mL FMT solution. Lastly, only five studies reported their compliance[31,33-35,44,45], and it was generally considered high because it ranged from 82.69% to 100%.

Effects of probiotics and prebiotics on the cognitive functions of healthy people

Regarding the healthy subjects, three studies showed no significant difference between probiotic and placebo groups[29,31,36]. In comparison, five studies showed a significant positive effect of the intervention on at least one of the performed cognitive tests[25,33,35,40,43].

In Benton *et al*[29], the healthy enrolled subjects ingested fermented milk containing *L. casei* Shirota daily for 3 wk. However, no significant differences between the probiotic and placebo groups were reported regarding episodic and long-term memory, assessed with the Wechsler Memory Scale test and the ability to remember the capitals of 30 countries. Moreover, the healthy people treated with *L. rhamnosus* supplement in Kelly *et al*[36] did not report any cognitive improvement, as assessed with the Paired Associates Learning, Attention Switching Task, Rapid Visual Information-Processing task (RVIP), Emotion Recognition Task and electroencephalography tests.

Considering the five studies reporting a significant cognitive improvement, Bagga *et al*[25] found that 4 wk administration of a multistrain probiotic increased Positive and Negative Affect Schedule (PANAS) score (paired with the response accuracy to unpleasant stimuli test) and showed the activation of the cingulum, pre-cuneum and cerebellum areas, involved in decision making and memory process.

Table 1 Summarizing of all selected studies

Ref.	Study design	Setting	Characteristics of the studied population	Number of participants (M/F)	Intervention	Comparison	Duration of intervention	Outcomes	Compliance
Agahi *et al*[23], 2018	RCT	Cities: Emam Ali, Golabchi, Miad, Barekat; Country: Iran	Patients with Alzheimer disease; Age: 65-90 yr; Control group: 80.57 ± 1.79 yr; Intervention group: 79.70 ± 1.72 yr	Total: 48; Control group = 23 (10/13); Intervention group = 25 (7/18)	1 capsule with *L. fermentum, L. plantarum, B. lactis* and 1 capsule with *L. acidophilus, B. bifidum, and B. longum* (3×10^9 CFU)	Placebo	12 wk	TYM	-
Akbari *et al* [24], 2016	RCT	Cities: Golabchi, Sadeghyeh; Country: Iran	Patients with Alzheimer disease; Age: 60-95 yr; Control group: 82.00 ± 1.69 yr; Intervention group: 77.67 ± 2.62 yr	Total: 60; Control group = 30 (24/6); Intervention group = 30 (24/6)	200 mL/d probiotic milk containing *L. acidophilus*, *L. casei*, *B. bifidum*, and *L. fermentum* (2×10^9 CFU each)	Placebo	12 wk	MMSE	-
Bagga *et al*[25], 2018	RCT	City: Graz Country: Austria	Healthy volunteers; Age: 20-40 yr; Control group (placebo): 27.25 ± 5.78 yr; No intervention group: 23.87 ± 4.97 yr; Intervention group: 28.27 ± 4.2 yr	Total: 45; Control group = 15 (9/6); No intervention group = 15 (7/8); Intervention group = 15 (7/8)	1 sachet/d with 3 g freeze-dried powder containing *L. casei* W56, *L. acidophilus* W22, *L. paracasei* W20, *B. lactis* W51, *L. salivarius* W24, *L. lactis* W19, *B. lactis* W52, *L. plantarum* W62 and *B. bifidum* W23(7.5×10^6 CFU/g)	Placebo or no intervention	4 wk	PANAS, SCL-90, ADS, LEIDS, RM task, ED task	-
Bajaj *et al*[26], 2014	RCT	City: Richmond, Virginia Country: United States	Patients with hepatic encephalopathy; Age: 18-65 yr; Control group: 58.5 ± 4.5 yr; Intervention group: 58.4 ± 3.8 yr	Total: 30; Control group = 16 (12/4); Intervention group = 14 (10/4)	*L. rhamnosus* GG (ATCC 53103)(> 50 billion CFU/gm)	Placebo	8 wk	NCT-A, NCT-B, DS, BDT	-
Bajaj *et al*[27], 2017	RCT	City: Richmond, Virginia Country: United States	Patients with hepatic encephalopathy; Mean age: 62 yr; Control group: 62.9 ± 9.8 yr; Intervention group: 64.5 ± 5.1 yr	Total: 20; Control group = 10 (10/0); Intervention group = 10 (10/0)	FMT units (90 mL total) instilled by enema and retained for 30 min	Standard of care	20 wk	EncephalApp-Stroop, PHES	-
Bajaj *et al*[28], 2019	RCT	City: Richmond, Virginia Country: United States	Patients with hepatic encephalopathy; Control group: 64.2 ± 6.2 yr; Intervention group: 63.3 ± 4.2 yr	Total: 20; Control group = 10 (8/2); Intervention group = 10 (8/2)	FMT capsules (550 µL of stool and buffer solution)	Placebo	20 wk	EncephalApp-Stroop, PHES	-
Benton *et al* [29], 2007	RCT	City: Swansea Country: Wales	Healthy volunteers; Age: 48-79 yr; Average age 61.8 ±7.3 yr	Total: 126 (51/75)	65 mL of milk drink containing *L. casei* Shirota (10^8/mL)	Placebo	3 wk	POMS, WMS, VFT, NART, Ability to recall the capital cities of countries	-
Buigues *et al* [30], 2016	RCT	City: Valencia Country: Spain	People with frailty syndrome; Age: 66-90 yr; Control group: 73.4 ± 1.8 yr; Intervention group: 74.2 ± 1.6 yr	Total: 50; Control group = 22 (6 /16); Intervention group = 28 (9/19)	7.5 g/d of Darmocare Pre® (Inulin 3375 mg, FOS 3488)	Placebo	13 wk	MMSE	-

Capitão *et al* [31], 2020	RCT	Cities: Swindon, Milton Keynes, London Country: United Kingdom	Healthy scholars; Age: 7-9 yr; Control group: 9.12 ± 1.02 yr; Intervention group: 8.54 ± 0.79 yr	Total: 35; Control group = 18 (12/6); Intervention group = 17 (12/5)	5.5 g/d of Bimuno (B-GOS, Lactose, Glucose, Galactose)	Placebo	12 wk	BAS-III, CogTrackTM battery, STAIC, MFQ	High (> 80%)
Ceccarelli *et al* [32], 2017	RCT	City: Rome Country: Italy	HIV-1 infected individuals; Median age: 48 (IQR: 38-54) yr; Intervention group: 45 (35-52.5) yr; Control group: 43 (38.2-53) yr	Total: 35; Control group = 26 (24/2); Intervention group = 9 (9/0)	Sachet containing *L. plantarum* DSM 24730 *S. thermophilus* DSM 24731, *B. breve* DSM 24732, *L. paracasei* DSM 24733, *L. delbrueckii subsp. bulgaricus* DSM 24734, *L. acidophilus* DSM 24735 *B. longum* DSM 24736, and *B. infantis* DSM 24737(450×10^9 bacteria)	Control group	24 wk	ROCF, RAVLT, STEP, VST, PVF, SVF, SPM, DS, CBTT, AAT, TMT A, TMT B	-
Chung *et al* [33], 2014	RCT	City: Jeonju Country: Korea	Healthy volunteers; Age: 60-75 yr; Control group: 64.50 ± 4.84 yr; Intervention group (500 mg): 64.50 ± 2.17 yr; Intervention group (1000 mg): 64.43 ± 4.47 yr; Intervention group (2000 mg): 66.56 ± 4.98 yr	Total: 36; Control group = 10 (4/6); Intervention group (500 mg) = 10 (9/1); Intervention group (1000 mg) = 7 (2/5); Intervention group (2000 mg) = 9 (5/4)	Daily doses of 500, 1000, or 2000 mg. of tablet containing *L. helveticus* IDCC3801	Placebo	12 wk	DS, SRT, VLT, RVIP, SCWT	> 70%
Inoue *et al*[34], 2018	RCT	City; Hyogo prefecture, Country: Japan	Healthy volunteers; Average age: 70.3 ± 3.1 yr; Control group: 70.9 ± 3.2 yr; Intervention group: 69.9 ± 3.0 yr	Total: 38; Control group = 18 (7/11); Intervention group = 20 (7/13)	Sachet containing lyophilised powder of *B. longum* BB536, *B. infantis* M-63, *B. breve* M-16V and *B.breve* B-3 (1.25×10^{10} CFU each)	Placebo	12 wk	MoCA, Modified flanker task, PHQ-9, GAD-7	> 99%
Hwang *et al* [35], 2019	RCT	City: Jeonju Country: South Korea	People with mild cognitive impairment; Age: 55-85 yr; Control group: 69.2 ± 7.00 yr; Intervention group: 68.0 ± 5.12 yr	Total: 100; Control group = 50 (14/36); Intervention group = 50 (20/30)	Mixture of fermented soybean powder and *L. plantarum* C29 (1.25×10^{10} CFU/g)	Placebo	12 wk	VLT, DS, ACPT	> 90%
Kelly *et al*[36], 2017	RCT	City: Cork Country: Ireland	Healthy volunteers; Age: 20-33 yr; Placebo/Probiotic group: 23.6 ± 0.97 yr; Probiotic/Placebo group: 25.64 ± 1.14 yr	Total: 29; Placebo/Probiotic group = 15 (15/0); Probiotic/Placebo group = 14 (14/0)	Active capsules contained corn starch, magnesium stearate, silicon dioxide and *L. Rhamnosus*(1×10^9 CFU)	Placebo	8 wk	MOT, PAL, AST, RVIP, ERT, Emotional Stroop	-
Kobayashi *et al* [37], 2019	RCT	City: Tokyo Country: Japan	People with memory complaints; Age: 50-80 yr; Control group: 61.6 ± 6.37 yr; Intervention group: 61.5 ± 6.83 yr	Total: 117; Control group = 58 (29/29); Intervention group = 59 (29/30)	1 capsule per day with *B. breve* A1 ($> 2 \times 10^{10}$ CFU)	Placebo	12 wk	RBANS, MMSE	-
Lew *et al*[38], 2019	RCT	Cities: Penang, Kubang Kerian Country: Malaysia	Stressed adults; Age: 18-60 yr; Control group: 32.1 ± 11.4 yr; Intervention group: 31.3 ± 10.8 yr	Total: 103; Control group = 51 (12/39); Probiotic group = 52 (12/40)	*L. plantarum* P8 (10^{10} CFU/sachet per day)	Placebo	12 wk	PSS-10, DASS-42, CBB	-
Ohsawa *et al* [39], 2018	RCT	Country: Japan	People with forgetfulness; 50-70 yr; Control group: 57.8	Total: 60; Control group = 29 (13/16); Intervention	One bottle per day (190 g per bottle) of a *L. helveticus*-fermented	Placebo	8 wk	RBANS, POMS	-

			± 5.9 yr; Intervention group: 58.5 ± 6.5 yr	group = 31 (13/18)	milk contained 2.4 mg of lactononadecapeptide				
Papalini *et al* [40], 2019	RCT	City: Nijmegen Country: The Netherlands	Healthy volunteers; Age:18-40 yr; Control group: 22 yr (SE = 0.5); Intervention group: 21 yr (SE = 0.4)	Total: 58; Control group = 29 (0/29); Intervention group = 29 (0/29)	2 g/d of powder diluted in water or milk containing *B. bifidum* W23, *B. lactis* W51, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *L. lactis* W19 *L. lactis* W58(5×10^9 CFU)	Placebo	4 wk	BDI, LEIDS-r, Emotional face-word Stroop task, Emotional face-matching paradigm, SCWT, DS, SECPT	-
Roman *et al* [41], 2018	RCT	City: Almeria Country: Spain	Fibromyalgia patients; Control group: 50.27 ± 2.03 yr; Intervention group: 55.00 ± 2.09 yr	Total: 31; Control group = 15 (2/13); Intervention group = 16 (1/15)	4 pills/d containing *L. Rhamnosus* GG®, *L. casei*, *L. acidophilus*, and *B. Bifidus* (6×10^6 bacteria per capsule)	Placebo	8 wk	MMSE, BDI, IGT, Two-choice Task	-
Rudzki *et al* [42], 2019	RCT	City: Bialystok Country: Poland	People with major depression; Control group: 38.90 (12) yr (SD); Intervention group: 39.13 (9.96) yr	Total: 60; Control group = 30 (10/20); Intervention group = 30 (7/23)	2 capsules/d containing *L. plantarum* 299v (10×10^9 CFU per capsule)	Placebo	8 wk	HAM-D 17, SCL-90, PSS-10, APT, RFFT, TMT A, TMT B, CVLT Stroop Test parts A and B	-
Smith *et al*[43], 2015	RCT	City: Cardiff Country: Galles	Healthy volunteers; Age: 19-30 yr; Mean age 23.0 yr	Total: 47 (19/28)	One sachet of Inulin per day (5 mg)	Placebo	4 h	Mood, Performance Tasks, Memory Tasks, Psychomotor Tasks, Selective Attention Tasks, Sustained Attention Task	-
Tamtaji *et al* [44], 2019	RCT	City: Kashan, Shahrekord Country: Iran	Patients with Alzheimer disease; Age: 55-100 yr; Control group: 78.5 ± 8.0 yr; Intervention group (Selenium): 78.8 ± 10.2 yr; Intervention group (Selenium + probiotic): 76.2 ± 8.1 yr	Total: 79; Control group = 26; Intervention group (Selenium) = 26; Intervention group (Selenium + probiotic) = 27	Selenium (200 μg/d) and probiotic containing *L. acidophilus*, *B. bifidum*, and *B. longum* (2×10^9 CFU/d each)	Placebo or only selenium (200 μg/d)	12 wk	MMSE	100%
Tamtaji *et al* [45], 2019	RCT	City: Kashan Country: Iran	Patients with Parkinson disease; Age: 50-90 yr; Control group: 67.7 ± 10.2 yr; Intervention group: 68.2 ± 7.8 yr	Total: 60; Control group = 30; Intervention group = 30	Probiotic containing *L. acidophilus*, *B. bifidum*, *L. reuteri*, and *L. fermentum* (each 2×10^9 CFU/g)	Placebo	12 wk	MDS-UPDRS	90%

AAT: Aachener Aphasia Test; ACPT: Auditory Continuous Performance Test; ADS: Allgemeine Depressionsskala; APT: Attention and Perceptivity Test; AST: Attention Switching Task; BAS-III: British Ability Scales III; BDI: Beck Depression Inventory; BDT: Block Design Test; CBB: Cogstate Brief Battery; CBTT: Corsi Block Tapping Test; CFU: Colony-forming unit; CVLT: California Verbal Learning Test; DASS-42: Depression Anxiety and Stress Scale questionnaire; DS: Digit Symbol Test; ED: Emotional Decision Making; ERT: Emotion Recognition Task; F: Female; GAD-7: Generalised Anxiety Disorder Questionnaire-7; HAM-D 17: Hamilton Depression Rating-17; IGT: Iowa Gambling Task; LEIDS: Leiden Index of Depression Severity; M: Male; MDS-UPDRS: Movement Disorders Society-Unified Parkinson's Disease Rating Scale; MMSE: Mini Mental State Evaluation; MoCA: Montreal Cognitive Assessment instrument; MOT: Motor Screening Test; NART: National Adult Reading Test; NCT-A: Number Connection Test A; NCT-B: Number Connection Test B; PAL: Paired Associates Learning; PANAS: Positive and Negative Affect Schedule; PHES: Psychometric Hepatic Encephalopathy Score; PHQ-9: Patient Health Questionnaire-9; POMS: Profile of Mood States; PSS-10: Perceived Stress Scale-10; PVFT: Phonological Verbal Fluency (PVF) test; RAVLT: Rey Auditory Verbal Learning Test; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status; RCT: Randomized controlled trial; RFFT: Ruff Figural Fluency Test; RM: Emotional Recognition Memory; ROCF: Rey-Osterrieth Complex Figure Test; RVIP: Rapid

Visual Information-Processing task; SCL-90: Symptom Checklist 90; SCWT: Stroop Color and Word Test; SECPT: Socially Evaluated Cold Pressor Test; SPM: Raven's Standard Progressive Matrices; SRT: Story Recall Test; STAIC: State-Trait Anxiety Inventory for Children; STEP: Test of Time and Weights Estimation; SVF: Semantic Verbal Fluency test; TMT-A: Trail Making Test A; TMT-B: Trail Making Test B; TYM: Test your memory; VFT: Verbal Fluency test; WMS: Wechsler Memory Scale MFQ: Children Mood and Feelings Questionnaire; VLT: Verbal Learning Test; VST: Visual Search Test.

Papalini *et al*[40] tested a probiotic multistrain mixture in women who underwent a stressful condition for 4 wk. The results showed that the trial reduced the unfavorable stress effect on working memory performance measured by the DS backward test. In addition, Chung *et al*[33] demonstrated a significant improvement in Verbal Learning Test, Story Recall Test, RVIP and Stroop Color and Word Test after 12 wk administration of *L. helveticus* in healthy subjects compared to placebo. Finally, Inoue *et al*[34] demonstrated that intervention with *Bifidobacterium spp.* for 12 wk, added to resistance training, significantly improved response accuracy and reaction time tests in healthy elderly subjects. Regarding the cognitive effects of prebiotic administration, the study conducted in healthy children by Capitão *et al*[31] reported that 12 wk GOS supplement only improved memory retrieval speed assessed with the CogTrackTM test battery. By contrast, Smith *et al*[43] investigated the acute effects of inulin intake on healthy volunteers and reported improving memory tasks, especially immediate free and delayed recall. No FMT intervention has been carried out in healthy subjects. Hence, although three of eight studies conducted in healthy subjects showed no significant difference between intervention and placebo groups, probiotics resulted were more effective in improving cognitive function than prebiotics.

Patients with different pathologies and the impact of probiotics/prebiotics/FMT on cognitive functions

The effects of probiotics, prebiotics, and FMT on cognitive functions were also assessed in different diseases, of which the most represented were hepatic encephalopathy (HE) and AD. Bajaj *et al*[26] conducted three studies on HE. In particular, the authors first investigated the effect of *Lactobacillus GG* administration on HE but did not report changes in cognition. However, they also treated HE patients with FMT *via* enema and reported a significant improvement in PHES and EncephalApp Stroop tests[27]. Moreover, Bajaj *et al*[28] evaluated the treatment with FMT capsules effects on HE patients, and they reported only a significant improvement in the EncephalApp Stroop test.

Regarding AD, Agahi *et al*[23] administered two different multistrain probiotic capsules to patients affected by severe disease for 12 wk, but no effect on TYM cognitive tests were reported. By contrast, Akbari *et al*[24] found that daily administration of probiotic milk enriched with *Lactobacillus spp.* led to a decline in Mini Mental State Evaluation (MMSE) score in AD patients compared to placebo. Moreover, Tamtaji *et al*[44] found that a probiotic and selenium co-supplement in AD patients was responsible for a significant increase in MMSE score. In addition, Hwang *et al*[35] found that people with mild cognitive impairment showed an improvement in a

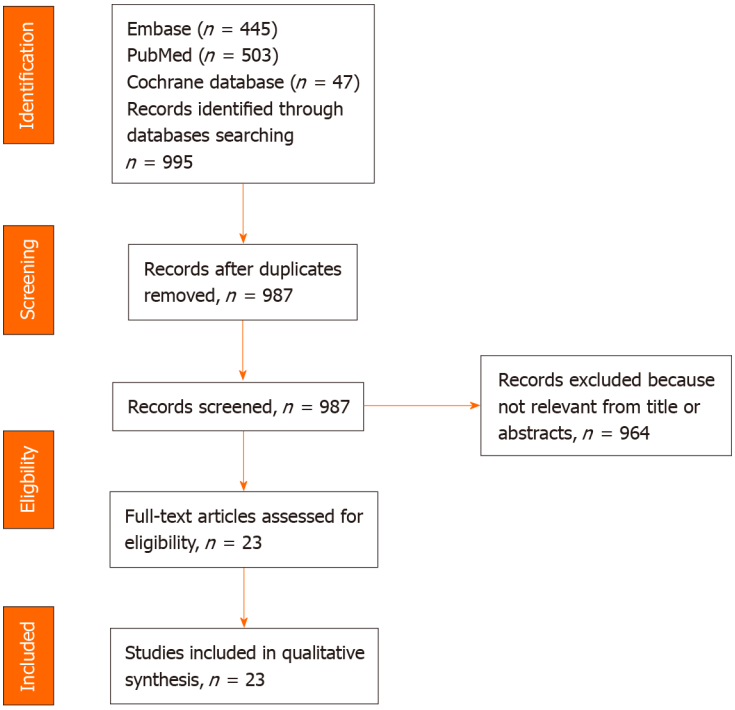

Figure 1 PRISMA flow diagram.

battery of tests related to verbal memory and attention domains after ingesting a mixture of *L. plantarum* C29 and fermented soybean powder. Finally, Lew *et al*[38] reported that daily administration of *L. plantarum* P8 for 12 wk in stressed adults led to a reduction of stress score and enhanced cognition and verbal learning memory, assessed through the CBB. Another study, conducted by Roman *et al*[41], explored the effect of a multispecies probiotic on fibromyalgia patients and found a significantly reduced number of impulsive choices. Moreover, Kobayashi *et al*[37] carried out a 12-wk treatment with *Bifidobacterium breve* A1 in elderly subjects with memory complaints, documenting a significant decline of total scores of both Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) and MMSE tests. Regarding patients affected by PD, Tamtaji *et al*[45] highlighted a favorable reduction of the Movement Disorders Society-Unified PD Rating Scale (MDS-UPDRS) after ingesting a probiotic mixture for 12 wk. In addition, Ohsawa *et al*[39] reported improved attention, coding, and delayed memory scores (assessed with RBANS) in people with forgetfulness after 8 wk intake of a *L. helveticus* fermented milk drink. Also, Rudzki *et al*[42] reported a significant improvement in CVLT and APT in people with major depression treated with *L. plantarum 299v*. Moreover, Ceccarelli *et al*[32] demonstrated a significant improvement in several cognitive functions in HIV-1 infected patients ingesting a multistrain probiotic for 24 wk (primarily in the following neurocognitive tests: Rey-Osterrieth Complex Figure, Rey Auditory Verbal Learning Test, Test of Time and Weights Estimation, Phonological Verbal Fluency Test, Corsi Block Tapping Test and Trail Making Test A). Finally, the only study which assessed the effectiveness of a prebiotic intake (inulin and fructooligosaccharides), conducted by Buigues *et al*[30] on elderly affected by frailty syndrome, the MMSE did not report significant cognitive improvement. As a result, while the FMT and especially probiotics played multiple beneficial effects on different cognitive functions of unhealthy subjects, the prebiotics' supplementation did not provide any cognitive improvement, maybe because of their short-term administration.

DISCUSSION

Dementia is a chronic, gradual, and progressive neurological disease that affects millions of people in both industrialized and rural countries. Cognitive decline and daily activities impairment limits patients' self-care and causes a severe burden to parents, friends, caregivers, and especially to the healthcare systems[46,47]. Increasing evidence suggests that the prevalence of dementia rises with age and is strongly

associated with other comorbidities, including AD and cardiovascular risk factors such as hypertension and hypercholesterolaemia[48].

Although the specific dementia pathogenesis is not yet understood and current therapies only attempt to counterbalance the disturbance, several studies recently highlighted the central role of the gut microbiota in brain health and the onset and persistence of neurodegenerative diseases[49,50]. Nevertheless, our systematic review of human RCTs reported contradictory results due to the diverse type, posology, and duration of interventions and the different responses of healthy or diseased people to the treatment.

In general, supplementation with probiotics and prebiotics determined the positive effects on healthy subjects. Five (63%) out[25,33,34,40,43] of the eight studies conducted in volunteers reported beneficial effects on the cognitive functions, while the other three[29,31,36] studies did not find any difference between the intervention and control groups.

Regarding the different evaluated patients, only 3 (20%)[23,26,30] of the 15 studies did not report an amelioration of cognitive functions for other possible reasons. For example, the probiotics' administration performed by Bajaj *et al*[26] probably did not last for a sufficient time to obtain cognitive improvement in patients with HE. In contrast, with 13 wk of prebiotics' supplementation, Buigues *et al*[30] did not observe effects on cognitive behaviour because MMSE does not represent a sensitive tool to detect the small changes in cognition that may occur after inulin and FOS supplementation. In addition, in the study conducted by Agahi *et al*[23], the 12 wk probiotic administration did not lead to cognitive amelioration in patients with AD; a probable explanation could be the enrollment of only patients with advanced disease.

Probiotic supplementation improves cognitive functions in many different diseases such as HIV[32], PD[45], fibromyalgia, major depression[42], AD[24,44], and other mild cognitive deficits[35,37-39]. Furthermore, studies evaluating the effects of FMT on patients with HE highlighted a significant amelioration in cognitive functionality[27,28]. It is well established that a balanced gut microbiota composition (eubiosis condition) plays a crucial role in our health; a dysbiotic status (meaning a reduced gut microbiota diversity) is related to many human gastrointestinal, immunological, and neurodegenerative diseases[51]. Concerning neurological impairments, recent findings elucidated the importance of the gut microbiota in the bidirectional communication between the central and enteric nervous systems, called the gut-brain axis[52]. Hence, the main factors responsible for intestinal dysbiosis such as stress, unbalanced diet, and drug abuse also determine an alteration of the gut-brain axis by causing a loss of epithelial integrity. The loss of this barrier functionality allows microbial-derived molecules to enter the systemic circulation, promoting endotoxemia, oxidative stress and low-grade inflammation responsible for the blood-brain barrier disruption[53,54] (Figure 2); these factors represent a signature for neurodegenerative disorders, especially AD. Consequently, given the importance of the intestinal barrier integrity for the prevention of neuroinflammation and brain damage, gut microbiota modulation by psychobiotics, namely beneficial bacteria (probiotics) or support for such bacteria (prebiotics) and FMT, represent an excellent strategy to restore the intestinal permeability and prevent the consequences of a leaky gut[55-57].

However, although several studies have highlighted the local beneficial effects of probiotics, prebiotics and FMT (*e.g.*, modification of the gut microbiota composition, strengthening of the gut epithelial barrier and modulation of the local (mucosal) immune system), they also exerted systemic effects, in particular on the central nervous system[58,60]. More specifically, recent studies have reported that the intestinal microbiota affects neurodevelopment and diverse brain functions by regulating the gut-brain axis, for example, by acting on the electrophysiological thresholds of the enteric nervous system neurons, which interact *via* neurotransmitters (adrenaline, noradrenaline, and acetylcholine) with the central nervous system[61].

Another important neuronal pathway in gut-brain communication involves the vagus nerve, and many effects of probiotics strains influence its activity[62]. Furthermore, since the gut houses the most extensive collection of lymphoid tissues in the human body and various intestinal immune cells can cross the blood-brain barrier, gut microbiota manipulation represents a key indirect route for communication between the gut microbiota and the central nervous system[63]. Intriguingly, specific probiotic formulations have also been shown an ability to stimulate the production of neurotransmitters (*e.g.*, GABA, serotonin, and dopamine) or are even microbially neuroactive. These microbial metabolites can trigger epigenetic signals on human brain genes involved in various complex networks or act as a ligand for specific human receptors[64].

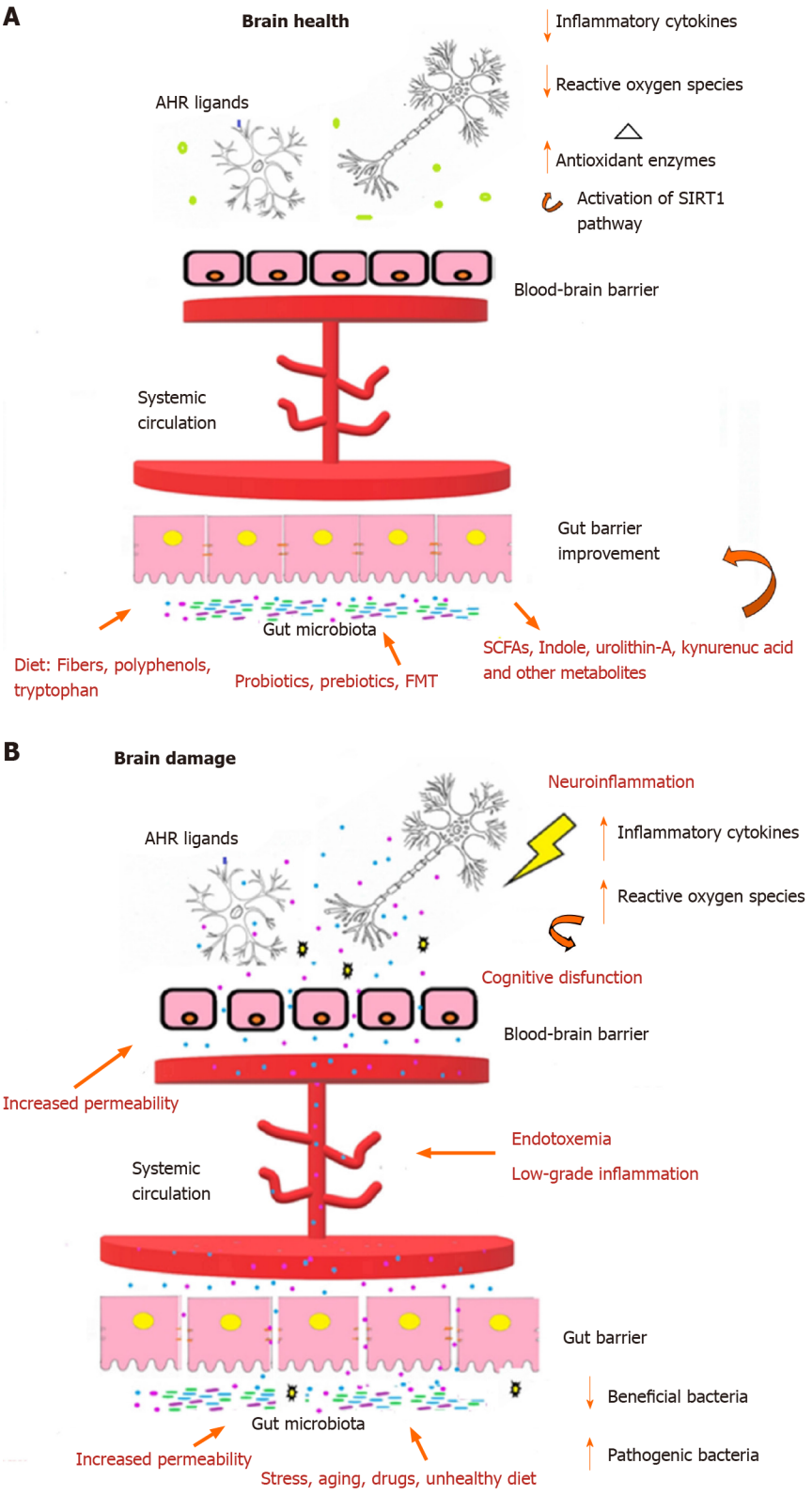

Figure 2 Gut-brain axis in eubiosis and dysbiosis condition. A: Eubiosis; B: Dysbiosis condition. AHR: Aryl hydrocarbon receptor; FMT: Fecal microbiota transplant; SCFA: Short-chain fatty acid; SIRT1: Sirtuin 1.

For instance, the probiotic activated Sirtuin 1 pathway, which regulates the brain antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, could favor cognitive improvements by preventing oxidative stress and deposition of beta-amyloid in the brain[65]. Even the modulation of kynurenine metabolism, the primary route for tryptophan catabolism, which is closely related to the structural and functional dynamics of the gut microbiota, could positively affect brain health[66]. Indeed, *in vivo L. plantarum* administration demonstrated a beneficial reduction of

kynurenines as most act as neurotoxic compounds[42,67,68]. Notably, although *L. plantarum* was administered to healthy or ill people in most of our selected RCTs, its positive effects have been probably underestimated because of the unknown impact of the other components of the probiotic formula that include it.

By contrast, indole-3-lactic acid (ILA) is an interesting neuroprotective tryptophan metabolite mainly produced by *Bifidobacterium spp*. acting as an aryl hydrocarbon receptor (AhR) agonist, expressed by intestinal and neuronal cells[69,70]. In detail, microbial agonists produced by *L. bulgaricus* and *L. reuteri* could activate microglia and astrocytes AhRs, suppressing pro-inflammatory signals and preventing neuronal damage[71-73]. Furthermore, the administration of some probiotics (especially *L. helveticus*, *L. casei* and *L. rhamnosus*) and prebiotics could also improve cognitive functions by stimulating the production of short-chain fatty acids as they enhance the transcription of the brain-derived neurotrophic factor that stimulates neuronal plasticity, protecting against neuroinflammation and neuronal apoptosis[74-78]. Moreover, the FMT represents a very promising strategy to re-establish gut eubiosis and improve cognitive functions. For instance, in transgenic mice, FMT significantly improved cognitive deficits, beta-amyloid accumulation, and neuroinflammation while reducing UPDRS score and tremor in people with Parkinson disease[79].

Finally, our recent study demonstrated that age-associated shifts of the microbiota have a detrimental impact on the central nervous system's protein expression and critical functions. Still, FMT represents an excellent strategy to restore a young-like microbiota and improve cognitive functions[80]. Therefore, although the modulation of intestinal microbiota represents a new precious therapeutic opportunity, it also shows some restrictions and risks. In particular, even if probiotics are generally considered safe and have many advantages such as a tolerated mode of administration (orally) and the possibility to integrate them with other pharmacological/non-pharmacological approaches, they displayed some limitations mainly due to potential side effects, especially in some patients (including immunocompromised people), or to their long-term safety[81]. Besides, even if probiotics can promote the production of several compounds such as lactic acid, bioamines, bile salts and other molecules that could play detrimental effects on the host, most of them are sold as dietetic supplements, and the regulatory agencies do not require safety studies in humans before their commercialization[82,83].

Although reported to be fairly safe in most clinical trials, FMT can be responsible for acute or prolonged adverse effects such as diarrhea, abdominal pain, nausea, headaches, and fatigue[84]. In particular, immunological concerns have been raised regarding safety assessments for both probiotics and the FMT because either indigenous or transient microorganisms could impact the immune system's functionality. Hence, the FMT application or the administration of probiotics to specific vulnerable populations and stressed or aged people, immunocompromised patients, newborns or pregnant women must be well evaluated to prevent microbial translocation and sepsis[85-87]. Moreover, the current literature lacks information about the long-term administration of probiotics; therefore, the possible horizontal transfer of antibiotic resistance genes favored by their supplementation cannot be excluded. Likewise, because stool contains thousands of microorganisms and a vast number of metabolites, FMT represents a constant risk of pathogens or commensals transfer to donors that may harmfully affect them[88].

CONCLUSION

As a final note, the different defects found in the evaluated studies highlighted some methodical limitations such as small sample sizes, the limited sampling time and the wide range of other cognitive tests. Supplementation of probiotics and FMT could represent a non-invasive successful strategy to restore gut eubiosis and enhance cognitive functions in healthy people and patients with different neurological/neurodegenerative diseases. Of course, further specific and clinical studies with numerous patients are needed to confirm this encouraging hypothesis.

ARTICLE HIGHLIGHTS

Research background

Due to the global population aging, cognitive impairments will affect approximately

115 million people by 2050. Since current therapies only attempt to counterbalance cognitive disorders, many recent studies recently highlighted the central role of the gut microbiota in brain health.

Research motivation

The pathogenesis of several cognitive disorders is still not fully understood; however, it has been recently established that a dysregulated gut-brain axis communication is associated with the onset and persistence of neurodegenerative diseases. Thus, gut microbiota manipulation could restore a functional gut-brain axis improving cognitive functions.

Research objectives

Since the evidence derived from human randomized clinical trials (RCTs) is currently limited, the main purpose of this systematic review was to detect the currently available RCTs, to define better the effects of probiotics, prebiotics, and fecal microbiota transplant (FMT) on cognitive functions.

Research methods

We systematically searched Embase, Medline/PubMed, Cochrane Library, central and clinicaltrials.gov databases with a combination of comprehensive terms related to cognition and gut microbiota manipulation. Then, we carefully reviewed and synthesized the data by types of study design and setting, characteristics of the studied population, kind of the intervention (strain type or mixture type, dosage and frequency of administration), control treatment, inclusion and exclusion criteria, follow-up duration, and cognitive or memory outcomes.

Research results

The analysis of the 23 included in our systematic review highlighted that, although the different type and posology of administration and the various cognitive tests and questionnaires adopted, both probiotics supplementation and FMT improved the cognitive functions in most of healthy people and patients affected by different neurological pathologies.

Research conclusions

The gut microbiota manipulation could represent a good strategy to counteract gut dysbiosis and so ameliorate cognitive dysfunction.

Research perspectives

The supplementation of probiotics and FMT could represent a non-invasive successful strategy to restore gut eubiosis and enhance cognitive functions in healthy people and patients with different neurological/neurodegenerative diseases.

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LETTER TO THE EDITOR

Impact of COVID-19 pandemic on the neuropsychiatric status of Wilson's disease

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Abstract

We have read with interest the Letter to the Editor by Drs. Zhuang and Zhong, who presented the clinical data of 68 patients with Wilson's disease (WD) who were admitted to the hospital before and during the coronavirus disease 2019 (COVID-19) pandemic, and appreciated their findings on hepatic and some extrahepatic manifestations. Nevertheless, given the strong impact of the pandemic on patients with neurological and psychiatric disorders, we would have expected a worsening of the psychiatric and/or neurological impairments in these patients. In contrast, according to the authors, these manifestations remained, somewhat unexpectedly, unchanged. This finding is in contrast with most of the current literature that highlights not only an increased incidence of mental health disorders in the general population but also an exacerbation of neurological and psychiatric symptoms in patients with chronic diseases, especially in those with pre-existing neuropsychiatric disorders, such as WD. Although the study was mainly focused on the hepatic features of WD patients taking anti-copper treatment, a generic and cumulative definition of neurological and psychiatric manifestations, as in this study, does not allow for further considerations. Future studies during and after the pandemic are necessary to clarify the real impact, either direct or indirect, of the COVID-19 pandemic on the neurological and psychiatric symptoms of WD patients.

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Core Tip: In the interesting letter by Drs. Zhuang and Zhong, the psychiatric and neurological manifestations of 68 patients with Wilson's disease who were treated with anti-copper therapy unexpectedly remained unchanged after the pandemic. Given the impact of the pandemic on patients with neurological and psychiatric disorders, a worsening in the severity or frequency of these manifestations could have been expected. The possible reasons underlying this finding, including the relatively small sample size, the effect of therapy, and the patients' resilience, are discussed.

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TO THE EDITOR

We have read with interest the Letter to the Editor by Zhuang and Zhong[1], who presented the clinical data of 68 patients with Wilson's disease (WD) who were admitted to the hospital before and during the coronavirus disease 2019 (COVID-19) pandemic in Guangzhou (China). Of note, none of the patients had COVID-19. As WD is a rare chronic systemic disease, the impact of the pandemic on the multiorgan clinical status of these patients is still unclear and, therefore, certainly worth investigating. Indeed, we have appreciated the findings that showed a marked shortage of medical resources for the clinical management of patients with WD during the pandemic, as well as the findings that patients who consistently took anti-copper medications showed no significant difference in hepatic and some extrahepatic features, although the incidence of their complications (especially those related to infections) significantly increased. For these reasons, we fully support the authors' recommendations to strictly adhere to the anti-copper therapy and closely monitor these patients to prevent complications[1].

However, given the strong psychological and socio-behavioral impacts of the pandemic on the clinical symptoms of different neuropsychiatric disorders[2,3], a worsening, or at least an increase in the frequency or severity, of psychiatric and/or neurological manifestations in WD patients could have been expected. Conversely, according to the authors, before the COVID-19 pandemic, 50 out of the 68 patients had neurological involvement, and three had psychiatric manifestations, which remained, somewhat unexpectedly, unchanged after the pandemic (49 and 4 out of the 68 patients, respectively)[1]. Although this study was mainly focused on the hepatic features of WD, only a generic and cumulative definition of neurological and psychiatric manifestations was adopted without additional specifications (*e.g.*, type, onset, severity, and duration), which did not allow for any further considerations; indeed, these apparently negative results were not discussed. Moreover, it was not specified how neurological and psychiatric manifestations were evaluated (*e.g.*, were specific scales used?), both at the study entry and at the end of the study. A more detailed stratification, for instance, by type of manifestation (such as cognitive or motor deficits, among the neurological aspects, and anxiety or mood disorders, among the psychiatric conditions), would have likely disclosed additional findings.

In this context, COVID-19, being the major infectious outbreak in the 21st century, has led to an unprecedented global hazard to mental health. A recent systematic review[4] on the impact of the pandemic on mental health in the general population found significantly higher rates of symptoms of anxiety (6.3%-50.9%), depression (14.6%-48.3%), post-traumatic stress disorder (7.0%-53.8%), psychological distress (34.4%-38.0%), and stress in general (8.1%-81.9%) in several countries worldwide, including China. Although a certain degree of heterogeneity was noted across the studies, the risk factors associated with these manifestations included, among others, a

younger age (≤ 40 years) and comorbid chronic or neuropsychiatric illnesses[4], such as WD.

On the other hand, the negative effect of the COVID-19 outbreak on mental health and health care services has been and will likely continue to be significant because of the unpredictability and uncertainty of the pandemic, the associated lockdowns, physical distancing, and other containment strategies, and the resulting economic breakdown[5]. Reasonably, as also observed in the Letter discussed here[1], the impact of the COVID-19 pandemic on the utilization of health care services, in terms of outpatient visits, hospital admissions, diagnostic exams, and therapeutic interventions, decreased by approximately one-third during the pandemic, with considerable variations and greater reductions among people with less severe illness[6]. Throughout the pandemic and even still, there has also been evidence of increased levels of relapse, in people with pre-existing mental health conditions and even in people with no previous history of a mental health disorder[7]. In particular, patients with pre-existing anxiety, depression, panic, delirium, psychosis, and suicidality appear to be extremely vulnerable[8].

Nevertheless, the matter is still debated, since it has also been observed that some individuals with severe mental illnesses, such as schizophrenia and affective disorders, may not report a worsening of symptoms, thus appearing to be resilient to the negative effects of the pandemic[9]. However, frequent assessments and periodic follow-up are needed to determine whether this resilience will persist as the pandemic progresses or after its end. In this context, in addition to the relatively small sample size, the potential effect of anti-copper therapy in this cohort, and the fact that none of the patients were affected by COVID-19, the patients' resilience might also represent a possible reason that supports the findings reported by Zhuang and Zhong[1]. However, their patients did not seem to be affected by severe psychopathologies, thus making this possibility less likely to justify the authors' conclusions. Moreover, although WD is a rare pathology, it is worth mentioning some methodological issues in the study[1] that may be appropriate and relevant for discussion. In particular, the relatively small sample size, the fact that not all patients were admitted to the hospital during the pandemic, and the possibility of the patients' resilience raise crucial questions that need to be addressed in the near future. Further multicenter prospective cohort studies, retrospective studies, and case-control studies on inpatients and outpatients with WD, both before and after anti-copper treatment, should be performed.

Less is known about the effects of the COVID-19 pandemic on neurological disorders, although a recent systematic review concluded that patients with pre-existing conditions (including those characterized by cognitive impairments or parkinsonism, which may be part of the clinical spectrum of WD) can develop exacerbation of their neurological symptoms, thus encouraging clinicians to be aware of this risk and to focus on its prevention and early management[10]. In this context, it is known that concomitant infections, especially infections of the respiratory and urinary tracts (such as those reported in the letter[1]), frequently worsen the symptoms and the course of several neurological diseases, including parkinsonism and dementia[11,12]. Therefore, the fact that the increased infection incidence detected in the study[1] did not induce an even transient worsening of the patients' clinical status remains to be explained or at least briefly commented on.

In conclusion, the study by Zhuang and Zhong[1] provides a valid clinical basis for the proper management of WD patients during the pandemic, thus representing an advance in this field of clinical and research interest. However, further independent multicenter studies during and after the pandemic are necessary to clarify the real impact, either direct (*i.e.*, infected patients) or indirect (*i.e.*, psychological and sociobehavioral consequences), of the COVID-19 pandemic on the neurological and psychiatric symptoms of WD.

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