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EVIDENCE REVIEW

# Pancreatic adenocarcinoma: Beyond first line, where are we?

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## Abstract

Pancreatic cancer is considered one of the most aggressive cancers, with an increasing incidence in recent years. To date, chemotherapy is still the standard of care for advanced metastatic disease, unfortunately providing only a slight advantage in terms of survival. The molecular and cellular characteristics of pancreatic cancer cells, as well as the cells that characterize the pancreatic tumour microenvironment, are the basis of the mechanisms of resistance to treatment. After progression during first-line treatment, few patients are eligible for second-line treatment due to the loss of performance status. To date, a clear survival advantage has not yet been demonstrated for second-line chemotherapy. Precision medicine could be the key to increasing responses to cancer treatment and finally impacting survival in this difficult-to-treat disease. In this review, we analyze current recommendations in the second-line setting and potential future prospects.

**Key Words:** Pancreatic adenocarcinoma; Second-line; Chemotherapy; Targeted therapy; Immunotherapy



**Core Tip:** The incidence of pancreatic ductal adenocarcinoma is increasing, with anticipation of a large impact on the population. Despite achieving a survival gain in first-line treatment in the last decade, to date, little has been achieved in second-line treatment. The molecular and genetic characteristics of this tumour represent a fundamental challenge for preclinical and clinical research. In this review, we illustrate current clinical practice in second-line treatment for advanced pancreatic adenocarcinoma and the research landscape of potential future prospects.

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## INTRODUCTION

Pancreatic cancer remains one of the deadliest malignancies, recording 432242 new deaths in 2018, with 458918 new pancreatic cancer cases reported globally[1]. Adenocarcinoma is the most common type of exocrine (non-endocrine) pancreatic cancer, accounting for over 90 percent of pancreatic cancer diagnoses. In most cases, it originates from the pancreatic ducts (ductal adenocarcinoma), in a smaller percentage of cases it can originate from the acini (acinar cell carcinoma). Rarer forms of pancreatic cancer are squamous cell carcinoma, adenosquamous carcinoma and colloid carcinoma. Despite advances in pancreatic cancer detection and management, the 5-year survival rate is still very low, only approximately 9%[2]. It is expected to become the second most common cause of cancer-related death by 2030[3]. Unfortunately, most cases are diagnosed in locally advanced or metastatic stages, for which chemotherapy remains the standard of care[4]. Progress in the treatment of pancreatic ductal adenocarcinoma (PDAC) has been very limited; in particular, gemcitabine (GEM) has been used as a monotherapy agent for first-line treatment for approximately 20 years. Subsequently, in 2011, there was a breakthrough in the treatment of metastatic PDAC (mPDAC) with the introduction of the FOLFIRINOX regimen [5-fluorouracil (5FU), folinic acid, irinotecan (IRI) and oxaliplatin (OX)] as a first-line standard of treatment[5]. However, this regimen is not suitable for all patients. Eventually, the combination of nab-paclitaxel and GEM (NabGem) also demonstrated an overall survival (OS) gain in mPDAC compared to GEM monotherapy[6]. However, no prospective randomized studies have demonstrated a benefit in terms of OS for a second-line treatment; moreover, there is currently no standard regarding the sequencing of treatments.

## CURRENT CLINICAL PRACTICE IN SECOND LINE MPDAC

### *Chemotherapy*

mPDAC is a biologically aggressive cancer that is often characterized by clinically evident disease progression during first-line treatment (pain, fatigue, anorexia, weight loss, constipation, fever, diabetic decompensation, *etc.*) with a deterioration of the patient performance status (PS) that limits subsequent treatments. Several complications can also arise, such as duodenal stenosis, obstruction of biliary stents and cholangitis, gastrointestinal bleeding and intestinal obstructions, which further limit the possibility of accessing second-line chemotherapy. In this context, it is not surprising that few data from large randomized trials are available. To date, there are no clear data on the superiority of a specific chemotherapy regimen due to the lack of adequate comparisons.

In advanced PDAC, the choice of which chemotherapy to use in the second-line setting basically depends on the treatments used in the first-line setting, residual toxicities (*e.g.*, peripheral neuropathy), patient PS, age and comorbidities. The ability of patients in different countries to access a specific treatment should also be considered due to the limitations of regulatory agencies.

Currently, in first-line treatment for patients with a good PS, Eastern Cooperative Oncology Group (ECOG) 0-1, two main regimens are indicated based on evidence of an OS benefit highlighted by randomized phase III trials: FOLFIRINOX and NabGem. In fact, the PRODIGE4/ACCORD11 trial showed the superiority of FOLFIRINOX over GEM in terms of OS (11.1 mo *vs* 6.8 mo), progression-free survival (PFS, 6.4 mo *vs* 3.3 mo) and the objective response rate (ORR, 31.6% *vs* 9.4%)[5], while in the Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT), NabGem showed superiority over GEM (OS 8.5 mo *vs* 6.7 mo, PFS 5.5 mo *vs* 3.7 mo, ORR 23% *vs* 7%, respectively)[6]. It is significant to consider how many patients received a subsequent therapy after progression on first-line therapy in these trials. In the PRODIGE4/ACCORD11 trial, second-line therapy was administered in approximately 50% of patients in both arms, while in the MPACT trial, second-line therapy was administered in 38% of patients in the NabGem group and in 42% of patients in the GEM group. Real-life data are very different; in fact, even in the best cases, they do not seem to include half of patients receiving second-line therapy. It is remarkable that patients

treated in high-volume centres, especially those aged < 65 years and with an ECOG PS of 0-1, receive more lines of therapy. According to Abrams *et al*[7], in the United States in 2015, approximately 56% of patients received second-line therapy and 22% received third-line therapy. The percentages reported in other recent databases are even lower: 38.2% in the United States[8], 33% in Sweden[9], and 44%-48% in British Columbia and Canada[10]. Higher percentages are reported in Austria, where the proportions of patients who had access to second- and third-line therapy were 62%-65% and 29%-37%, respectively[11].

The choice of subsequent treatment has to consider the chemotherapy drugs received in the first line. Therefore, there are two main scenarios: after a first-line treatment with GEM-based chemotherapy, the advice of the main guidelines is to choose a 5FU-based chemotherapy; in the case of a front line therapy with a 5FU-based scheme, the indication is a GEM-based therapy[12,13]. The choice between a multidrug combination regimen and monotherapy depends on the patient's PS (ECOG 0-1 or 2, respectively). A summary of the current possible options is reported in Figure 1. The study by Taieb *et al*[14] evaluated first-line and second-line treatment regimens and their geographic variation across European countries between 2014 and 2016, highlighting that the most common first-line treatments were FOLFIRINOX (35.6%), the first choice in France and in the United Kingdom; NabGem (25.7%); and GEM monotherapy (20.5%). Overall, GEM was the most frequently used second-line therapy (27.1%), followed by NabGem (17.8%), FOLFOX [5FU+ leucovorin (LV) + OX, 17.6%] and 5FU monotherapy (16.7%)[14]. It should be noted that nab-paclitaxel beyond the first line is not approved in all countries, and at that time, pegylated liposomal IRI (Nal-IRI) was not yet available.

Pancreatic cancers with specific molecular characteristics, such as microsatellite instability, fusion of the *NTRK* gene, and *BRCA 1-2* mutations, require a separate discussion. In fact, currently, it is recommended by National Comprehensive Cancer Network guidelines to evaluate at least these three genetic features, but unfortunately, this is not accessible yet for everyone in several countries.

For a better understanding of the data available in the literature, we considered the following possible scenarios: (1) Second-line chemotherapy after treatment with FOLFIRINOX; and (2) Second-line chemotherapy after GEM-based regimens (Figure 1).

### *Second-line chemotherapy after treatment with FOLFIRINOX*

There is no clear consensus on the second-line treatment after progression to FOLFIRINOX since no prospective randomized trials have been conducted in this setting. The choice is generally a GEM-based treatment, which could be GEM monotherapy or a GEM-based therapy. Table 1 summarizes the main second-line trials and their results, divided according to the type of study.

**GEM in monotherapy:** Only a series of retrospective studies have evaluated the efficacy of GEM as a second-line monotherapy after FOLFIRINOX failure[15,16]. The analysis conducted by Viaud *et al*[17] showed a median OS with GEM of 3.7 mo [95% confidence interval (CI): 2.5-5.2], a median PFS of 2.1 mo (95%CI: 2.0-2.6) and a disease control rate (DCR) of 40%, highlighting that age at diagnosis and PS were independently associated with OS in a multivariate analysis [hazard ratio (HR) of 1.86; $P = 0.0055$ and 2.42; $P < 0.0001$, respectively] and suggesting that GEM is beneficial for patients with a good PS. A multicentre retrospective study in the same setting showed an ORR of 11% and a clinical benefit of 44% for patients, regardless of their previous response to the first-line treatment, concluding that some patients benefit from a second-line treatment[18].

**GEM based treatment:** No randomized trials have evaluated the efficacy of the NabGem combination as second-line therapy. Zhang *et al*[19] published retrospective data collected from a total of 146 patients treated with FOLFIRINOX as the first-line treatment. Of those, 30 received the NabGem combination, 8 received GEM as monotherapy, and 22 received best supportive care (BSC). The median PFS and OS were 3.61 mo and 5.69 mo in the NabGem group and 2.51 mo and 3.82 mo in the GEM monotherapy group, respectively. In a second retrospective study[10], the percentage of patients receiving NabGem compared to GEM alone was different depending on the region considered and the respective possibility for reimbursement[20]. In this study, the OS outcomes favour the NabGem combination regardless of funded access to the second-line combination. The efficacy of the combination in the second-line setting was confirmed in a third multicentre retrospective analysis, although without a comparison with GEM alone[21]. A prospective study showed that the DCR with

**Table 1 Studies of second-line treatment in metastatic pancreatic ductal adenocarcinoma**

| Ref. | Type of study | Patients (*n*) | 1st-line regimen | 2nd-line regimen | Median 2nd-line OS (mo) | Median 2nd-line PFS (mo) | 2nd-line ORR (%) | 2nd-line DCR (%) |
|---|---|---|---|---|---|---|---|---|
| Pelzer *et al*[35], 2011 | Phase III | 46[1] | GEM monotherapy | OFF; BSC | 4.8; 2.3 | - | - | - |
| Oettle *et al*[36], 2014, CONKO-003 | Phase III | 160 | GEM monotherapy | OFF; FF | 5.9; 3.3 | 2.9; 2.0 | - | - |
| Gill *et al*[37], 2016, PANCREOX | Phase III | 108 | GEM-based (approximately 75% monotherapy) | mFOLFOX6; FU/LV | 6.1; 9.9 | 3.1; 2.9 | 13.2; 8.5 | 60; 63.8 |
| Wang-Gillam *et al*[37], 2016, NAPOLI-1 | Phase III | 417 | GEM-based[2] | Nal-IRI; FU/LV; Nal-IRI + FU/LV | 4.9; 4.2; 6.1 | 2.7; 1.5; 3.1 | 6; 1; 16 | 44; 24; 52 |
| Chung *et al*[29], 2018 | Phase II | 48 | GEM-based | mFOLFIRINOX | 9.0 | 5.8 | 18.8 | 62.5 |
| Tsavaris *et al*[33], 2005 | Phase II | 30 | GEM | OX 50 mg/mq + FU/LV (1-h iv infusion), weekly | 6.25 | - | 23.3 | 53.3 |
| Pelzer *et al*[32], 2009 | Phase II | 37 | GEM | OFF | 5.5 | 3.0 | 6 | 49 |
| Yoo *et al*[34], 2009 | Phase II | 61 | GEM-based | mFOLFIRI.3; mFOLFOX | 3.9; 3.5 | 1.9; 1.4 | 0; 7 | 23; 17 |
| Zaniboni *et al*[49], 2012 | Phase II | 50 | GEM ± platinoid | FOLFIRI | 5 | 3.2 | 8 | 36 |
| Chung *et al*[29], 2018, SWOG S1115 | Phase II | 137 | GEM-based | Selumetinib+ MK-2206; mFOLFOX | 3.9; 6.7 | 1.9; 2.0 | 1.7; 8 | 22.4; 30.6 |
| Portal *et al*[22], 2015 | Prospective cohort | 57 | FOLFIRINOX | NabGem | 8.8 | 5.1 | 17.5 | 58 |
| Zaanan *et al*[47], 2014 | Prospective cohort | 46 | GEM/FOLFIRI.3 in FIRGEM trial | FOLFOX | 4.3 | 1.7 | 0 | 36 |
| Wainberg *et al*[45], 2020 | Meta-analysis | 454 | GEM-based | FOLFOX; Nal-IRI | 6.3; 6.1 | - | - | - |
| Sonbol *et al*[51], 2017 | Meta-analysis | 895 | GEM-based | FPOX; FPIRIFP | FPIRI *vs* FP: HR OS 0.7, PFS 0.64; FPOX *vs* FP: HR OS 1.0, PFS 0.81 | | | |
| Citterio *et al*[52], 2018 | Meta-analysis | 1587 | GEM-based | FP, OX or IRI-based | Most effective IRI-based regimens (results cannot be translated into the table) | | | |
| Rahma *et al*[43], 2013 | Systematic analysis | 1503 | GEM-based | GEM + platinum; FPOXBSC | 6.0; 5.7; 2.8 | 4; 2.9; - | - | - |
| Petrelli *et al*[53], 2017 | Systematic analysis | - | GEM-based | OX-based; IRI-based | 5.3; 5.5 | 2.9; 2.7 | 11.9; 8.7 | 41.1; 29.4 |
| Berk *et al*[48], 2012 | Comparative | 85 | GEM-based | FOLFOX4; XELOX | 5.8; 4.9 | 3.7; 3.7 | 17; 18 | 43; 59 |
| Zhang *et al*[19], 2018 | Retrospective | 146 | FOLFIRINOX | NabGem; Gem alone | 5.69; 3.82 | 3.61; 2.51 | - | - |
| Chae *et al*[21], 2020 | Retrospective | 102 | FOLFIRINOX | NabGem | 9.8 | 4.6 | 8.5 | 73.6 |
| Viaud *et al*[17], 2017 | Retrospective | 96 | FOLFIRINOX | GEM monotherapy | 3.7 | 2.1 | - | 40 |
| Gilabert *et al*[18], 2017 | Retrospective | 72 | FOLFIRINOX | GEM monotherapy | - | 2.5 | 11 | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pointet *et al*[38], 2020 | Retrospective | 137 | NabGem | FOLFOX; FOLFIRI; FOLFIRINOX | 3.5; 9.7; 6.1 | 2; 6.6; 3.4 | 0; 9.5; 6.3 | 29.2; 61.9; 50 |
| Lee *et al*[41], 2020 | Retrospective | 120 | GEM-based | FPOX; FP | 7.04; 7.43 | 2.89; 3.81 | 6.4; 5.4 | 52.6; 59.5 |
| Neuzillet *et al*[50], 2012 | Retrospective | 63 | GEM ± platinoid | FOLFIRI | 6.6 | 3.0 | 7.9 | 39.7 |
| Kieler *et al*[24], 2019 | Retrospective | 52 | GEM-based | Nal-IRI + FU/LV | 6.79 | 3.84 | 19.2 | 46.2 |

[1]The trial was prematurely stopped due to insufficient accrual.
[2]Approximately 45% of gemcitabine alone and 55% in combination. About 30% of patients had received ≥ 2 previous lines for metastatic disease, with 45% of patients pretreated with fluorouracil/leucovorin-based regimens. GEM: Gemcitabine; DCR: Disease control rate; BSC: Best supportive care; OFF: Oxaliplatin, folinic acid and 5-fluorouracil; 5FU: 5-Fluorouracil; OX: Oxaliplatin; LV: Leucovorin; FF: Folinic acid and 5-fluorouracil; Nal-IRI: Liposomal irinotecan; FOLFIRINOX: 5-Fluorouracil, folinic acid, irinotecan and oxaliplatin; FOLFOX: 5-fluorouracil + leucovorin + oxaliplatin; FPOX: Fluoropyrimidine and oxaliplatin-based regimens; HR: Hazard ratio; OS: Overall survival; NabGem: Nb-paclitaxel and gemcitabine; FP: Fluoropyrimidine; IRI: Irinotecan.

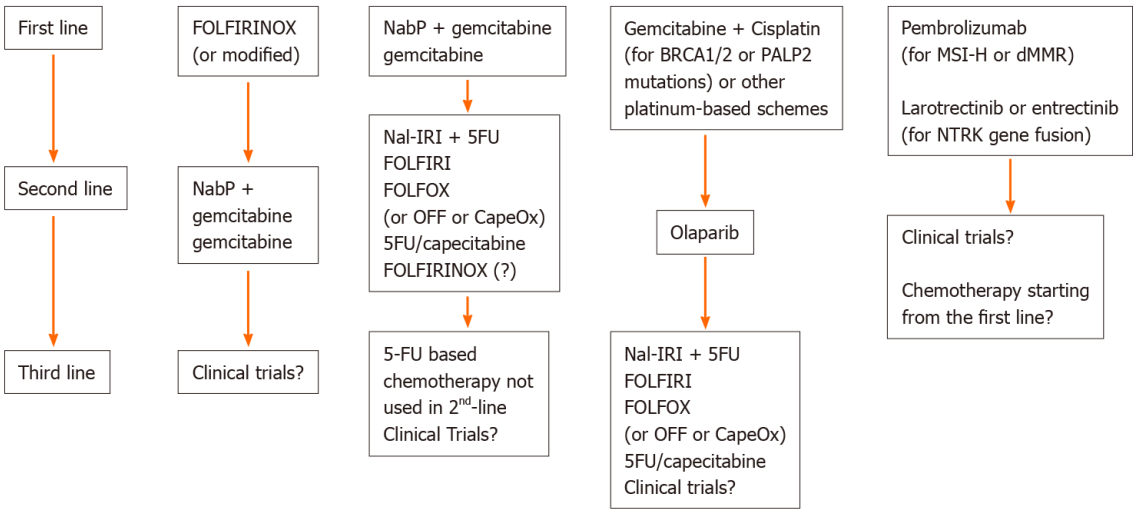


**Figure 1 Current therapeutic possibilities for metastatic pancreatic ductal adenocarcinoma.** NabP: Nab-paclitaxel; Nal-IRI: Nanoliposomal irinotecan; 5FU: 5-Fluorouracil; FOLFIRI: 5-Fluorouracil + irinotecan; FOLFOX: 5-fluorouracil + leucovorin + oxaliplatin; OFF: Oxaliplatin + 5-fluorouracil + folinic acid; CapeOx: Capecitabine + oxaliplatin; MSI-H: Microsatellite instability high; dMMR: DNA mismatch repair deficiency; Clinical trials?: Evaluate the availability of clinical trials suitable for the patient.

NabGem was 58% (ORR 17.5%), OS was 8.8 mo (95%CI: 6.2-9.7) and the PFS was 5.1 mo (95%CI: 3.2-6.2)[22].

To date, there are no second-line treatment recommendations after progression on the FOLFIRINOX scheme, and the use of GEM alone or in combination with nabpaclitaxel is generally dictated by patient characteristics and by the possibility of reimbursement in individual countries.

### *Second-line chemotherapy after treatment with GEM based combination therapy*

For patients previously treated with GEM-based regimens, the main international guidelines recommend 5FU-based therapies, which include FOLFIRI, Nal-IRI+5FU, OX, folinic acid and 5FU (OFF), FOLFOX or CapeOX and monotherapy with 5FU or capecitabine.

**Nal-IRI ± 5FU/LV:** Nal-IRI + 5FU/LV is the regimen with the most evidence and therefore a higher degree of recommendation[13]. This indication comes from the results of the NAPOLI-1 study, which compared 5FU/LV alone *vs* monotherapy with Nal-IRI or the combination of 5FU/LV + Nal-IRI in 417 patients with mPDAC and a Karnofsky PS ≥ 70 who were previously treated with GEM-based therapy[23]. In particular, 12% of patients received GEM-based therapy in the adjuvant, neoadjuvant, or locally advanced setting; 56% had received one previous line of metastatic treatment; and 32% had received two or more lines of metastatic treatment. It should be emphasized that few patients received NabGem in the first-line setting since the

GEM combination is preferred in current clinical practice, and 43% of patients had already received previous 5FU-based therapy (10% IRI and 32% platinum). Patients in the 5FU/LV + Nal-IRI group achieved a longer OS than patients in the 5FU/LV group (6.1 mo *vs* 4.2 mo; $P$ = 0.012, HR: 0.67); however, no significant difference in OS was observed between the 5FU/LV and Nal-IRI monotherapy groups (4.2 mo *vs* 4.9 mo; $P$ = 0.94, HR: 0.99).

These data were confirmed by a retrospective study that included 52 patients[24] and a similar Korean study[25]. However, in some countries, including Italy, this combination is not approved due to the methodological limitations of the study, such as the heterogeneity of the patient population, the study design without a comparison with the classic FOLFIRI regimen, and the inclusion of patients pretreated with 5FU or IRI[26].

**Fluoropyrimidine and OX-based regimens:** The efficacy and safety data of second-line treatment with FOLFIRINOX are based on retrospective analyses[27,28] and on some phase II studies[29,30]. In the single-arm multicentre phase 2 study performed by Chung *et al*[29] (48 patients) of modified FOLFIRINOX (IRI 120 mg/m$^2$ and OX 60 mg/m$^2$), the ORR, DCR, median PFS and OS were 18.8%, 62.5%, 5.8 mo and 9.0 mo, respectively, with significant toxicity (neutropenia grade 3 or 4 rates of 64.6%, febrile neutropenia 16.7%). A highly toxic triplet therapy is not very suitable for second-line palliative treatment in patients with a non-optimal PS and is reserved for only a few cases. Furthermore, in a recent real-world analysis, triplet therapy with FOLFIRINOX did not seem to have an advantage over sequential chemotherapy with FOLFIRI-FOLFOX regimens[31].

For the other OX-based regimens, the data are controversial. Based on the promising results of some phase II studies[32-34], three main phase III trials have been conducted[35-37]. In the CONKO-003 trial, 160 patients were randomized to receive FF (folinic acid 200 mg/m$^2$ followed by a continuous IV infusion of fluorouracil 2000 mg/m$^2$ over 24 h on days 1, 8, 15, and 22 every 42 d) or OFF (FF and OX 85 mg/m$^2$ IV administered before FF on days 8 and 22). Compared to FF, OFF achieved a significant increase in both OS (5.9 *vs* 3.3 mo) and PFS (2.9 *vs* 2 mo)[36]. In phase III by Pelzer *et al*[35], OFF compared to BSC significantly prolonged OS (4.82 mo *vs* 2.30 mo, respectively) despite the premature closure for insufficient accrual (only 46 patients) due to the difficulty of clinicians and patients accepting BSC[35]. However, these results of the superiority of OFF over FF and BSC were not confirmed by the phase III PANCREOX trial[37]. In particular, in this study, the addition of OX to FF (in the mFOLFOX6 scheme) did not translate into an increase in OS; in contrast, it seemed detrimental (6.1 mo *vs* 9.9 mo) at the expense of increased toxicity.

In the literature, different retrospective trials and reviews have dealt with the same topic, with discordant results[38-47].

A comparative study evaluated the XELOX and FOLFOX schemes, highlighting their comparable results in terms of efficacy and toxicity profile[48].

**IRI and 5FU-based regimens (fluoropyrimidine IRI):** The use of second-line FOLFIRI in patients who progressed on first-line therapy of GEM and platinum (cisplatin or OX) was evaluated in a prospective multicentre study[49]. Among the 50 patients enrolled, four partial responses (8%) were observed with disease stability in 28% of patients, while PFS and OS were 3.2 mo and 5.0 mo, respectively. Similar results were obtained from another study that evaluated FOLFIRI after progression on GEM and platinoids[50]. Unlike the previous study, patients ($n$ = 63) could receive more than one treatment line in the metastatic setting. In particular, most patients had received two previous lines. DCR was achieved in 25 patients (39.7%; partial response: $n$ = 5, stable disease: $n$ = 20) with FOLFIRI. The median time to progression (TTP) was 3.0 mo, and the median OS was 6.6 mo. An ECOG PS of 2 was significantly associated with a poor TTP and OS, limiting the efficacy of FOLFIRI to patients with a good PS (PS 0-1).

Some meta-analyses have concluded that fluoropyrimidine (FP) IRI (FPIRI) is superior to FP and OX-based regimens (FPOX) after first-line treatment with gem-based chemotherapy. In particular, Sonbol *et al*[51] collected randomized controlled trials comparing FP monotherapy *vs* FPOX or FPIRI and showed that FPOX or FPIRI improved PFS compared with single-agent FP, but only FPIRI reported an OS advantage. Similarly, in the network meta-analysis by Citterio *et al*[52] and the meta-analysis by Catalano *et al*[40], FPIRI seemed superior to FPOX in terms of OS. Conversely, in the systematic review of 24 studies by Petrelli *et al*[53], FPOX and FPIRI were associated with a similar efficacy, with a pooled ORR, DCR, PFS and OS of 11%, 37.9%, 2.87 mo and 5.48 mo, respectively.

In conclusion, in patients with preserved PS (ECOG PS 0-1), without relevant comorbidities, it is reasonable to propose a second-line treatment with a 5FU-based or GEM-based treatment, depending on the first-line treatment used. Within the 5FU-based regimens, any residual toxicities of the first-line treatment can lead to choose a scheme rather than another. For example, if the patient has residual neurotoxicity (*e.g.*, from Nab-paclitaxel) the choice could be FOLFIRI or Nal-IRI-5FU; if he has diarrhea or bone marrow toxicity, FOLFOX. However, some treatments, such as NabGem or Nal-IRI, are not reimbursed for the second-line in all countries, thus inevitably influencing the choice of treatment.

### *Targeted therapy*

The introduction of increasingly accurate techniques for molecular sequencing and a better understanding of the pathogenetic role of genes related to PDAC have led to the drafting of numerous clinical trials to study potential targeted treatments in chemorefractory disease. Studies in the literature suggest that the use of precision medicine can have a substantial effect on survival in patients affected with PDAC and that molecular-guided treatments targeting oncogenic drivers promise potential developments in clinical practice[54]. Despite countless studies, to date, few biologic treatments have been approved for advanced PDAC. In particular, the FDA (Food and Drug Administration)- and EMA (European Medicines Agency)-approved targeted drugs for second-line treatment are erlotinib, larotrectinib and entrectinib. Olaparib is approved for maintenance after response to a first-line platinum-containing agent[55].

**Erlotinib:** The approval of erlotinib, an EGFR (epidermal growth factor receptor) TK inhibitor, in combination with GEM comes from a phase III study that demonstrated a statistically significant, albeit modest, improvement in survival in PDAC compared to GEM alone[56]. These data have been confirmed in other prospective[57] and retrospective studies[58].

**Larotrectinib and entrectinib:** Fusions involving *NTRK1*, *NTRK2* and *NTRK3* lead to the expression of chimeric rearrangements in tropomyosin receptor kinases (TRKs) A, B, and C, respectively, with constitutively active kinase function. TRK fusions are oncogenic drivers in numerous cancer histotypes, including pancreatic cancer, albeit in a very low percentage of cases, approximately 0.34%[59]. A peculiarity of the studies that evaluated TRK inhibitor drugs is that the efficacy of the specific treatment on a genomic alteration is evaluated independent of the tumour histology. No randomized trials have been conducted, but the high ORR that exceeded the predetermined minimum of the investigators (30%), which was 75% for larotrectinib and 57% for entrectinib, led to the approval of these drugs. However, data from studies of these two drugs are not comparable given the heterogeneity of the study populations involved.

The approval of entrectinib for solid tumours with *NTRK* gene fusions is based on the results of three clinical trials: ALKA-372-001, STARTRK-1[60] and STARTRK-2 (NCT02568267). An integrated analysis of the following phase I and II studies included a total of 54 patients with *NTRK* fusion-positive advanced solid tumours for a total of 19 different histotypes[61]. The median follow-up was 12.9 mo and showed 50% partial responses and 7% complete responses. This response to treatment has been maintained over time with a median duration of response of 10 mo and a good toxicity profile.

The approval of larotrectinib is based on data from three multicentre, open-label, single-arm clinical studies: LOXO-TRK-14001 (NCT02122913), SCOUT (NCT02637687) and NAVIGATE (NCT02576431). A pooled analysis of these studies by Hong *et al*[62] included 55 patients treated with larotrectinib. The ORR was 79% (95%CI: 72-85) in 153 evaluable patients, with a 16% complete response rate and a good safety profile.

Given the clinical benefit, even considering the low prevalence of *NTRK* fusions in patients with pancreatic cancer and the lack of easy access to Next Generation Sequencing services, patients should be tested at diagnosis for such gene alterations to guide treatment decisions as well as for gaining access to potential clinical trials.

Unfortunately, in real life clinical practice it is not possible to require such molecular insights in all patients with mPDAC due to the cost sustainability. The lack of access to these drugs in different countries represents the current gap between what precision medicine for mPDAC could be in the future and current clinical practice in different oncology contexts. The use of resources and the high costs of oncological treatments will be an increasingly important topic in the near future and it is inevitable to take this into account.

### *Immunotherapy*

Immunotherapy has changed the natural history of various cancer pathologies, especially melanoma and lung cancer, providing results in terms of increased OS in other neoplastic pathologies, such as cancer of the head and neck, bladder cancer, renal carcinoma, Merkel cell carcinoma and triple-negative breast tumours. However, to date, immune checkpoint inhibitors (ICI) have not shown any efficacy in controlling advanced PDAC, either in monotherapy[63,64] or in combination with chemotherapy[65,66]. Several actors are known to be responsible for the response/resistance mechanisms to ICI. Among these actors, the ability of the tumour to express antigens recognizable by the cells of the immune system and the characteristics of the tumour microenvironment in which the balance of immuno-sensitizing/immuno-suppressive factors is in favour of the latter[67]. In particular, PDAC are characterized by the presence of an abundant desmoplastic stroma composed of fibroblasts, extracellular matrix, immune cells and stellate cells. Immune cells infiltrating this stroma are mostly represented by tumour-associated macrophages, myeloid-derived suppressor cells and Treg cells, with very few effector T cells. Numerous trials are underway aimed at converting the pancreatic tumour microenvironment from immunosuppressive to immunosensitive[68,69]; however, to date, there is no indication for second-line immunotherapy treatments in chemorefractory disease. A PD-1 (programmed cell death protein 1) ICI, Pembrolizumab, has been approved by the FDA for diseases with microsatellite instability, regardless of tumour site[70]. However, this indication has not yet been approved by EMA, and in Europe, it is therefore not possible to prescribe immunotherapy in this setting outside of clinical trials.

## FUTURE DIRECTIONS

As seen from current clinical practice in second-line treatment of mPDAC, there are fundamental open questions. These questions include therapeutic possibilities for treatment after progression on TRK inhibitors in TRK fusion-positive cancers, an increase in targeted therapies, and overcoming the immuno-resistance of metastatic pancreatic disease.

### *Therapeutic possibilities for treatment after progression on TRK inhibitors in TRK fusion-positive cancers*

Patients may acquire resistance to first-generation *TRK* inhibitors; however, to date, the mechanisms of resistance to *TRK* inhibitor drugs are not known, and the only secondary resistance mechanism identified is the acquisition of targeted mutations in the *NTRK* kinase domain of the oncogenic fusion. Currently, several trials of newer molecules, such as LOXO-195[71] and TPX-00005[72], have been performed to evaluate the efficacy of targeted therapies after progression on *TRK* inhibitors with very promising results. Such molecules could become the second-line standard in the future after the failure of first-generation *TRK* inhibitors in *TRK* fusion-positive pancreatic adenocarcinoma.

### *Increase of targeted therapies*

Despite the high ORR of *TRK* inhibitor drugs, unfortunately, the percentage of PDAC patients susceptible to this targeted treatment is extremely low. Furthermore, identification of the *BRCA* mutation allows the prescription of olaparib in maintenance after a response to a first-line platinum-based treatment, so there is currently no possibility of second-line targeted treatment in mutated *BRCA* patients[73].

To date, there are few data on the efficacy of targeted treatments in advanced PDAC; however, this deficiency should not discourage clinicians from requiring a genomic study in these patients. Indeed, knowledge of the prospective genomic profile can predict the response to chemotherapy treatment[74]. Furthermore, these data could be useful for the enrolment of second-line patients in a clinical trial. Research is currently progressing by targeting the recombination deficits of DNA as well as considering the driver genes in pancreatic carcinogenesis. Alongside drugs that target pancreatic tumour cells, there is a large amount of research addressing the peculiar tumour microenvironment that is partly responsible for the poor response to cancer treatments of advanced PDAC (Figure 2).

Regarding tumours with recombination deficiency of DNA repair, research is also underway to determine the extent of the cancer risk in patients with the so-called

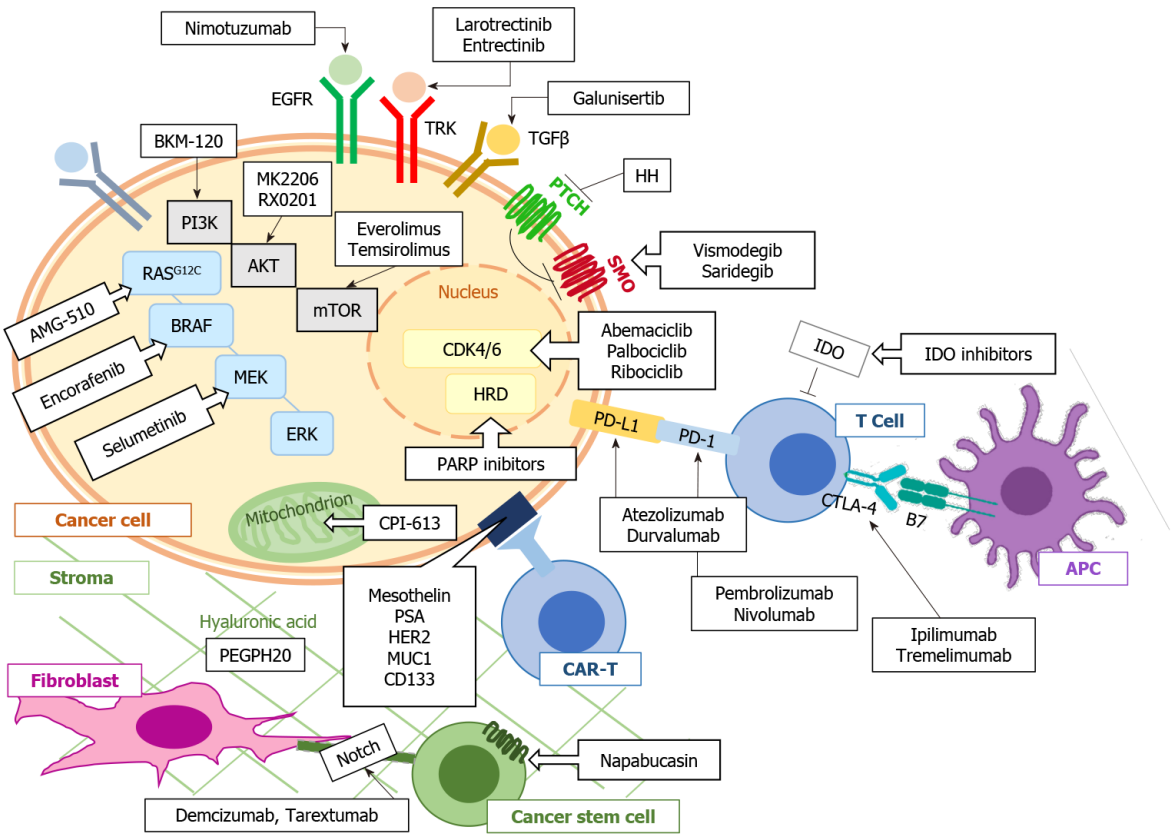


**Figure 2 Main targeted drugs under study for patients with advanced pancreatic ductal adenocarcinoma.** EGFR: Epidermal growth factor receptor; TGFβ: Transforming growth factor β; APC: Antigen presenting cells; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death protein ligand 1; CAR-T: Chimeric antigen receptor T cells.

'bracness' phenotype, or rather the genetic alterations that result in a defect in homologous recombination repair, mimicking the loss of *BRCA1* or *BRCA2*. Among these mutations is the *PALB2* mutation, which occurs in 3%-4% of familial pancreatic cancer cases[75,76]. Most studies of *PARP* inhibitors are conducted as maintenance after a response or stability after treatment with a platinum-based first line, *i.e.*, the current indication for olaparib. However, studies have also been conducted on the second line in patients pretreated and in progression after a first-line chemotherapy treatment[77-79]. However, in this setting, the data are currently conflicting, promising for olaparib and rucaparib and not significant for veliparib. Nevertheless, phase 3 studies are lacking.

Considering the major driver genes in pancreatic carcinogenesis, pancreatic tumours are characterized in most cases by activating mutations in *KRAS* (> 90%) and loss-of-function mutations in *TP53* (50%) and *CDKN2A* (80%). Several studies are underway with the aim of targeting such drivers; however, to date, the potential therapeutic target genes are limited to *KRASG12C* and *CDKN2A*, which are found in only a small percentage of patients. AMG 510 is a novel small molecule that specifically and irreversibly inhibits *KRASG12C* and shows antitumour activity when administered as monotherapy in pretreated patients[80,81]. Since the loss of p16INK4a is a standard feature in *KRAS*-driven PDAC, pharmacological specific inhibition of CDK4/6 represents a possible targeted treatment. However, monotherapy treatment with CDK4/6 inhibitors does not appear to be remarkably effective for pancreatic cancer[82]. It has therefore been hypothesized that the activity of CDK4/6 inhibitors can be exploited by combination therapies, such as mTOR inhibitors or chemotherapy[83]. Numerous clinical trials are currently underway (Table 2).

PI3K/Akt signalling is one of the most deregulated signalling pathways in cancer, including PDAC, and has a mediating role of the cellular signalling not only for tumour cells but also for stromal cells. Indeed, *KRAS* activates various signalling pathways of downstream effectors, including the PI3K pathway, which can, in turn, be activated by different signal transduction pathways linked to various growth factor receptors. In the last decade, there has been considerable interest in molecules inhibiting the PI3K/Akt-mediated transduction pathway, including PDAC[84,85]. One

**Table 2 Main ongoing targeted therapy studies (clinicaltrial.gov) for advanced pancreatic adenocarcinoma**

| Treatment | Target | Phase of study | Setting |
|---|---|---|---|
| Ribociclib plus trametinib; NCT02703571 | CDK4/6 | Phase I/II trial, open label single arm | Advanced or metastatic pancreatic cancer and *KRAS*-mutant colorectal cancer |
| Palbociclib + the PI3K/mTOR inhibitor, gedatolisib; NCT03065062 | CDK4/6 | Phase I, open label single arm | Advanced squamous cell lung, pancreatic, head and neck and other solid tumours |
| Abemaciclib in combination with the TGF-β inhibitor galunisertib or other agents; NCT02981342 | CDK4/6 | Phase II, open label, randomized | Previously treated metastatic pancreatic ductal adenocarcinoma |
| BKM120 + mFOLFOX6; NCT01571024 | PI3K | Phase I, open label, single arm | Advanced solid tumours including metastatic pancreatic cancer |
| Metformin + Gemcitabine + Erlotinib; NCT01210911 | PI3K | Phase II, randomized, placebo controlled | Locally advanced or metastatic pancreatic cancer |
| Capecitabine + Cetuximab + Everolimus; NCT01077986 | mTOR | Phase I/II, open label, single arm | Metastatic pancreatic cancer |
| Temsirolimus; NCT00075647 | mTOR | Phase II, open label, single arm | Locally advanced or metastatic pancreatic cancer |
| MK2206 + Fluorouracil + Oxaliplatin + Selumetinib; NCT01658943 | Akt | Phase II, open label, randomized | Metastatic pancreatic cancer |
| RX-0201 + Gemcitabine; NCT01028495 | Akt | Phase II, open label, single arm | Metastatic pancreatic cancer |
| Gemcitabine ± nimotuzumab; NCT02395016 | EGFR | Phase III, prospective, randomized, controlled, double-blind | Locally advanced or metastatic pancreatic cancer |
| MRTX849 (inhibitor of KRAS G12C) + TNO155 (inhibitor of SHP2); NCT04330664 | KRASG12C | Phase I/II, open label, non-randomized | Advanced or metastatic cancer with a KRAS *G12C* mutation |
| AMG 510 Monotherapy; NCT03600883 | KRASG12C | Phase I/II, open label, non-randomized | *KRAS* p.G12C mutant advanced solid tumours |
| Gemcitabine + M7824 (TGF-β ligand trap); NCT03451773 | TGF-β | Phase I/II, open label, single arm | Locally advanced or metastatic pancreatic cancer |
| FFX *vs* CPI-613 + mFFX; NCT03504423 | CPI-613 | Pase III, open-label randomized | Mtastatic adenocarcinoma of the pancreas |

TGF-β: Transforming growth factor β.

of the major challenges contributing to the suboptimal response to PI3K inhibitor drug monotherapies is the development of resistance mechanisms. Therefore, in this case, the current standard is the identification of new targeted combination therapies[86]. Table 2 reports the current ongoing clinical trials targeting the phosphoinositide signalling cascade for the treatment of pancreatic cancer.

The transformation of growth factor beta (TGF-β) signalling regulates cell proliferation and plays a fundamental role in the process of metastasis, angiogenesis and escape from immune surveillance. Several TGF-β inhibitory molecules are being studied, including oral inhibitors of the TGF-β receptor kinase, such as galunisertib (LY2157299), which specifically downregulates SMAD2 phosphorylation, blocking the activation of the canonical pathway[87], and trabedersen (AP 12009), a TGF-β2-specific antisense phosphorothioate oligodeoxynucleotide[88]. For example, data from a phase Ib study using galunisetinib in combination with second-line durvalumab suggests possible second-line activity of the combination[89].

CPI-613 is a new anticancer drug that selectively targets the altered form of mitochondrial energy metabolism in cancer cells, compromising the activity leading to apoptosis of cancer cells. Following the promising results of Phase I and II studies[90], a Phase III study is underway to compare this combination of FOLFIRINOX and CPI-613 with FOLFIRINOX alone.

Numerous other potential targets have been studied, such as c-KIT, VEGFR, and RET. Unfortunately, both masatinib, an anti-cKIT tyrosine kinase inhibitor, and vandetanib, an anti-VEGFR2, -RET, and -EGFR tyrosine kinase inhibitor, have failed to demonstrate a benefit over standard therapy[91,92].

A possible explanation for the failure of targeted therapies is the adaptive response to drug inhibition, for example, through the blockage of downstream signalling and the activation of other signalling transduction pathways. The future is trending towards the identification of combinations of treatments, with the aim of overcoming resistance mechanisms with an acceptable toxicity profile. Alongside this trend, there

is the need to identify predictive molecular markers of response to treatment.

### *Overcome immuno-resistance of metastatic pancreatic disease*

As previously reported, the initial enthusiasm for immunotherapy in advanced pancreatic cancer waned due to the not very encouraging data from early clinical trials. However, the improved understanding of resistance mechanisms has led to further clinical studies aiming to overcome the immuno-resistance of the pancreatic tumour microenvironment.

To date, data from clinical trials that evaluated the combination of ICI drugs in second-line treatment are not promising. The study conducted by O'Reilly *et al*[93] that evaluated the efficacy of the combination of durvalumab and tremelimumab in patients who progressed to first-line FP or GEM did not yield the desired results.

For the association of immunotherapy drugs with chemotherapy, the data seem to be encouraging; however, numerous association trials are also underway with cancer vaccines, adoptive T cell transfer, and direct targeted treatments in the tumour microenvironment (JAK/STAT inhibitors, CSF1R blockers, BTK inhibitors)[94].

Again, there is a lack of factors that allow us to predict the response to treatment; greater knowledge of the individual genetic characteristics together with the molecular characteristics of the disease could in the future lead to a broader selection of patients for immunotherapy treatments.

## CONCLUSION

Treatment of patients with mPDAC has improved in recent years thanks to the introduction of more effective chemotherapy regimens in the first-line setting. Consequently, the proportion of patients who are candidates for second- and third-line regimens is increasing. However, to date, chemotherapy remains the second-line standard of care, and neither personalized medicine nor immunotherapy has in fact provided important positive results in the treatment of pancreatic cancer. There are many ongoing studies aiming to overcome the multiple resistance mechanisms to treatment; however, the key to overcoming these mechanisms and providing personalized medicine to patients who have progressed to a first line of treatment is far from being identified. The small improvements shown by ongoing clinical trials could be considered a first step in what could be the future of treatment for advanced pancreatic cancer.

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*REVIEW*

# Non-alcoholic fatty liver and chronic kidney disease: Retrospect, introspect, and prospect

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## Abstract

With the growing prevalence of obesity and diabetes in the United States and across the world, a rise in the overall incidence and prevalence of non-alcoholic fatty liver disease (NAFLD) is expected. The risk factors for NAFLD are also associated with the development of chronic kidney disease (CKD). We review the epidemiology, risk factors, genetics, implications of gut dysbiosis, and specific pathogenic mechanisms linking NAFLD to CKD. Mechanisms such as ectopic lipid accumulation, cellular signaling abnormalities, and the interplay between fructose consumption and uric acid accumulation have led to the emergence of potential therapeutic implications for this patient population. Transplant evaluation in the setting of both NAFLD and CKD is also reviewed. Potential strategies for surveillance and management include the monitoring of comorbidities, the use of non-invasive fibrosis scoring systems, and the measurement of laboratory markers. Lastly, we discuss the management of patients with NAFLD and CKD, from preventative measures to experimental interventions.

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**Core Tip:** Patients with non-alcoholic fatty liver disease (NAFLD) are at higher risk for the development of chronic kidney disease (CKD) than the general population. The prevalence of mutual comorbidities in addition to direct pathogenic mechanisms linking NAFLD to the development of CKD can explain this finding. With the breadth of data linking NAFLD to CKD, there are minimal options for treating this patient population. Regardless, we have presented strategies that can be implemented at various levels including surveillance, preventative, and management level.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of chronic liver disease ranging from steatosis on one end to fibrosis and cirrhosis on the other end[1]. NAFLD and non-alcoholic steatohepatitis (NASH) are the hepatic manifestations of metabolic syndrome (MetS), which is a driving force for a multitude of comorbidities, such as insulin resistance, cardiovascular disease (CVD), chronic kidney disease (CKD), obstructive sleep apnea (OSA), as well as increased malignancy risk[2]. While NASH is the second leading indication for liver transplantation (LT), it is expected that NASH will overtake hepatitis C virus (HCV) as the leading cause, given the efficacy of direct-acting antiviral therapy[3]. A recent epidemiological study has already confirmed a downward trend for HCV-related LT in the United States[4].

NAFLD is tightly linked to underlying insulin resistance and is associated with other comorbidities related to MetS[5]. Growing evidence suggests that NAFLD is a risk factor for CKD[6] due to shared metabolic risk factors[7]. Of note, several studies have shown an association between the severity of NASH and CKD[8-11]. Interestingly, a meta-analysis of 33 studies showed that diabetes status and metabolic risk factors had no impact on the positive correlation between the severity of NASH and CKD[12], suggesting a possible unique pathogenic link between NAFLD and CKD irrespective of their shared metabolic risk factors. We review the genetic, epidemiologic, and pathogenic links between NAFLD and CKD in addition to potential preventative and management strategies.

## PREVALENCE OF CKD IN NAFLD AND POTENTIAL PREDISPOSING RISK FACTORS

Two meta-analyses and a retrospective cohort analysis suggest that the incidence and prevalence of CKD increase in patients with NAFLD compared to patients without NAFLD (Table 1). In all analyses, the magnitude and direction of effects remained unaffected by diabetes status, even after adjustment for other risk factors[12-14]. Moreover, the association was stronger in patients with advanced fibrosis or decompensated cirrhosis as compared to compensated cirrhosis. The studies that were included in these major meta-analyses defined advanced fibrosis was defined by histological parameters, imaging findings, and/or elevations in the NAFLD fibrosis score (NFS). Of note, among 42 studies included in these two meta-analyses, only 13 ($n$ = 2205) utilized liver histology, which is the gold standard in diagnosing NAFLD[15]. The majority of the studies established diagnosis of NAFLD *via* abdominal ultrasound, liver enzyme elevation [including serum gamma-glutamyl transferase (GGT)

**Table 1 Incidence and prevalence of chronic kidney disease in patients with varying degrees of non-alcoholic fatty liver disease severity**

| Ref. | Year | *n* | NAFLD diagnostic modalities | Conclusion(s) |
|---|---|---|---|---|
| Musso *et al*[12]. A meta-analysis of 33 studies | 2014 | 63902 | Liver biopsy, abdominal ultrasound, elevated liver enzymes | (1) 20 cross-sectional studies: Nearly two-fold increased risk of CKD in patients with NAFLD (OR 2.12, 95%CI 1.69-2.66); (2) 11 longitudinal studies: 1.8-fold increased risk of CKD in patients with NAFLD (HR 1.79, 95%CI 1.65–1.95); and (3) advanced fibrosis associated with increased prevalence (OR 5.20, 95%CI 3.14-8.61) and incidence (HR 3.29, 95%CI 2.30-4.71) of CKD in patients with NAFLD |
| Mantovani *et al*[13]. A meta-analysis of 9 studies | 2018 | 96595 | Abdominal ultrasound; FLI; serum GGT | Incidence of CKD: (1) 1.4-fold increased long-term risk (HR 1.37, 95%CI 1.20–1.53) in patients with NAFLD with a median follow-up period of 5.2 years; and (2) 1.5-fold increased risk (HR 1.50, 95%CI 1.25-1.74) in patients with severe NAFLD (defined as NFS ≥ -1.455 or serum GGT ≥ 109 U/L) |
| Park *et al*[14]. Retrospective Cohort with Propensity Score Matching (1:3) | 2019 | 262619 | ICD-9 | Incidence of CKD: 1.4-fold increased risk (aHR 1.41; 95%CI, 1.36-1.46) in patients with NAFLD after adjusting for demographics, baseline covariates, and ACEi/ARB use; Risk of incident CKD increases as the severity of NAFLD increases: (1) compensated cirrhosis (aHR, 1.47; 95%CI 1.36-1.59); and (2) decompensated cirrhosis (aHR, 2.28; 95%CI 2.12-2.46) |

NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; HR: Hazard ratio; FLI: Fatty liver index; GGT: Gamma glutamyl transferase; NFS: NAFLD fibrosis score; CI: Confidence interval; OR: Odds ratio; ACEi: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker; ICD-9: International classification of disease-9.

elevation], or using international classification of disease-9 code.

Previous review articles estimated that the prevalence of CKD was 20% to 55% in patients with NAFLD, whereas the prevalence of CKD in patients without NAFLD was 5% to 30%[16,17]. However, most of these reviews evaluated the same pool of data[8,9,11,18-22], which were also included in the two meta-analyses mentioned above. Our conclusions were based on studies that were published before 2015 as several more recent studies did not use histology or imaging for NAFLD diagnosis[23-26].

Many non-hepatic and hepatic risk factors are associated with CKD in those with NAFLD.

### Non-hepatic risk factors

There is minimal data on non-hepatic risk factors to predict which patients will go on to develop CKD. However, there are a few studies outlined below to identify which patients may be at higher risk (Table 2).

### Smoking

Current cigarette smoking is associated with CKD or death from end-stage renal disease. Mainstream cigarette smoke includes over 4000 compounds, and nicotine is one of many biologically stable and active compounds present in tobacco. Nicotine causes kidney damage by modulating α7nAChR, NLRP6 inflammasome, ER stress, and autophagy[27,28]. Studies examining the relationship between smoking and NAFLD are lacking; however, in a cohort study of 1525 CKD patients who underwent repeated health check-up examinations over 10 years, the decline in estimated glomerular filtration rate (eGFR) associated with NAFLD was greater in current smokers, hypertensive patients, or those with lower eGFR at baseline had greater age- and sex-adjusted decline in eGFR[29].

### Diabetes

Around one-third of patients with NAFLD have impaired renal function and its prevalence in patients with NAFLD is dependent on the severity of liver disease and presence of diabetes mellitus[30]. The development of NAFLD in patients with diabetes appears to be an important event in its natural history predisposing these patients to a higher risk for developing CKD. Type 2 diabetes mellitus (T2DM) increases the risk of serious NASH and advanced fibrosis in patients with NAFLD[31,32]. Patients with T2DM or type 1 diabetes mellitus and NAFLD are at an increased risk of developing CKD compared to diabetics without NAFLD[20,33,34]. Despite accumulating evidence for NAFLD as a driver for CKD, the shared common risk factors make it difficult to isolate diabetes as an independent risk factor for CKD in NAFLD patients.

**Table 2 Summary of studies assessing non-hepatic risk factors for chronic kidney disease in patients with non-alcoholic fatty liver disease**

| Ref. | Risk factor(s) | Year | *n* | Comparison | Findings |
|---|---|---|---|---|---|
| Önnerhag *et al*[147] | Older age | 2019 | 120 | Biopsy-proven NAFLD *vs* non-NAFLD | Higher prevalence of CKD in patients ≥ 55 years old |
| Targher *et al*[20] | Diabetes mellitus | 2008 | 2103 | NAFLD and T2DM *vs* T2DM only | Patients with NAFLD and T2DM independently associated with increased risk of CKD (OR 1.87; 95%CI 1.3-4.1, $P$ = 0.020) |
| Targher *et al*[33] | Diabetes mellitus | 2010 | 301 | NAFLD and T1DM *vs* T1DM only | Patients with NAFLD and T1DM independently associated with increased risk of CKD |
| Jang *et al*[29] | Elevated baseline eGFR, HTN, and current smoking | 2018 | 1525 | NAFLD *vs* Non-NAFLD | The decline in eGFR associated with NAFLD appeared to be stronger among patients who were current smokers, hypertensive, and lower eGFR at baseline |

NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; T2DM: Type 2 diabetes mellitus; OR: Odds ratio; CI: Confidence interval; T1DM: Type 1 Diabetes Mellitus; eGFR: Estimated glomerular filtration rate; HTN: Hypertension.

### *Hypothyroid*

Proper thyroid function is implicated in renal blood flow, glomerular and tubular function, electrolyte homeostasis, hepatic lipid metabolism, and fatty acid beta-oxidation[35]. Hypothyroidism can cause NAFLD through fat accumulation, while hyperthyroid can cause NAFLD through reactive oxygen species formation[36]. Additionally, the prevalence of hypothyroidism increases for each 10 mL/min/1.73 $m^2$ decrement in eGFR[37], and patients with hypothyroidism were more than 2 times likely to have NAFLD and 4 times more likely to have NASH[38].

### *Hepatic risk factors*

**NAFLD-related advanced fibrosis:** Patients with NAFLD-related advanced fibrosis are more likely to have CKD compared to patients with NAFLD but without advanced fibrosis[39]. The risk of albuminuria increases with the severity of NAFLD-related advanced fibrosis, according to a 2017 study of 1763 Chinese diabetic patients[40]. After adjusting for common CKD risk factors such as diabetes and other metabolic comorbidities, advanced fibrosis but not steatosis was associated with a higher risk of albuminuria (OR: 1.52; 95%CI: 1.02-2.28; $P$ = 0.039). In a 2019 study of 594 patients with T2DM, significant liver fibrosis as detected by elastography (LSM ≥ 7.0/6.2 kPa) was independently associated with a higher risk of CKD (adjusted OR: 3.6, 95%CI: 1.3-10.1; $P$ = 0.01) in addition to CVD and other microvascular complications[41]. Increased liver stiffness as detected by transient elastography is a predictor of CKD in patients with ultrasound-diagnosed NAFLD[42]

In a 12-year prospective cohort, patients with non-obese NAFLD had a higher risk of developing CKD than patients with obese NAFLD[43]. A recent study has noted that the risk of developing CKD is higher in metabolically unhealthy non-obese NAFLD patients than their counterparts with metabolically healthy status defined by the lack of metabolic risk factors (*i.e.* diabetes mellitus, low High-density lipoprotein, hypertriglyceridemia, arterial hypertension)[44].

**Pathophysiology:** CKD secondary to fatty liver is thought to be due to systemic low-grade inflammation[45], which may involve upregulation of the nuclear factor-κB (NF-κB) pathway[45,46]. As discussed earlier, there is circumstantial evidence to suggest that patients with NASH-related advanced fibrosis have an increased prevalence of CKD. Progression of NASH may be partly mediated by the altered renin-angiotensin-aldosterone system due to CKD has also been proposed as a mechanism for NAFLD progression[47]. Although direct pathogenic links between NAFLD and CKD seem to be confounded by common metabolic comorbidities, novel mechanisms have been described.

**Insulin resistance**: Increased adiposity leads to increased free fatty acids and pro-inflammatory cytokine release that causes systemic insulin resistance (IR), which is an established mediator of NAFLD. IR is further exacerbated by the progression of NAFLD, leading to atherogenic dyslipidemia and further release of inflammatory cytokines resulting in CKD as shown in animal models[48]. Proinflammation occurs through the NF-κB and Jun-N-terminal kinase (JNK) pathways; activation of adipose-specific JNK pathways has been shown to cause insulin resistance[48,49]. As NAFLD

progresses to NASH, the inflammatory component is neutrophil-predominant and can cause systemic endothelial dysfunction (Figure 1)[50,51]. Notably, IR leads to increased production of very-low-density lipoprotein and endoplasmic reticulum stress, both of which can cause podocyte damage in glomeruli[52]. These latter two mechanisms have been linked to proteinuria and subsequent hastening of CKD[53,54].

**Ectopic lipid accumulation:** In animal models, a high fat/fructose diet resulted in increased urinary albumin excretion, elevated transaminases, and increased incidence of liver tumors when compared to a standard diet. Microscopically, lipid deposition leads to accelerated hepatorenal pathologies, suggesting that intracellular lipid accumulation may link NAFLD to CKD[55]. When treated with fenofibrate, slower intracellular lipid accumulation was noted in co-incidence with slower progression of renal and hepatic pathologies[55].

**Wnt signaling abnormalities:** Alterations in cellular pathways critical for homeostasis play an important role in the development of CKD in patients with NAFLD. Specifically, abnormalities in the Wnt (named as a fusion of the Drosophila gene wingless and its vertebrate homolog, integrated) signaling pathway have been linked to lipid accumulation, chronic inflammation, and fibrosis in the development of both NAFLD and CKD[56].

**Sterol regulatory element-binding proteins**: Sterol regulatory-element binding proteins are activated in a nutrient-rich (*i.e.*, anabolic) state that leads to insulin-signaling and increased endoplasmic reticulum stress, which can cause increased lipogenesis and hepatosteatosis. These changes cause the progression of other metabolic phenomena such as CKD and MetS[57].

**Fructose consumption and uric acid accumulation:** Fructose intake has been linked to hepatorenal injury *via* uric acid accumulation by altering the gut microbiome (Figure 2)[45,58]. Patients with a normal body mass index (BMI) and elevated serum uric acid levels (> 10 mg/dL) have an increased prevalence of MetS when compared to patients with a serum uric acid < 6 mg/dL[59], which is corroborated by other studies[60-62]. An increase in serum uric acid levels is also associated with an increase in the incidence of NAFLD[63]. In patients with NAFLD, elevated uric acid levels are known to be pathogenic in CKD progression[42,64]. These studies suggest that MetS, NAFLD, and CKD are interconnected through elevated serum uric acid levels[65].

Uric acid stimulates fructokinase, which sensitizes hepatocytes to fructose metabolism, subsequently leading to fat deposition in the liver, thereby explaining the link between elevated uric acid and NAFLD[66]. Elevated uric acid levels in animal models lead to glomerular hypertension and tubulointerstitial fibrosis, two processes that preclude the development of CKD[64]. Decreased urate clearance in CKD patients may further exacerbate this pathology. Interestingly, xanthine oxidase inhibitors are currently being tested in patients with CKD to monitor for disease progression in the CKD-FIX[67].

**Gut dysbiosis:** Changes in the gut microbiome play a role in the pathogenesis of NAFLD and CKD[45]. Dietary conditions such as increased fructose intake and vitamin D deficiency are shown to cause dysbiosis, which may directly lead to low-grade inflammation responsible for the development of NAFLD and CKD[45]. Dysbiosis and subsequent microbial fermentation lead to increased production of uremic toxins indoxyl sulfate and p-cresyl sulfate, which correlate directly with the progression of CKD[68]. The liver cytochrome P450 enzymes are directly regulated by these uremic toxins derived from alterations in gut microbial metabolism, hence the gut-liver-kidney axis[69]. Animal models have also shown the gut microbiota's ability to metabolize choline into trimethylamine N-oxide (TMAO), which is considered both nephrotoxic and hepatotoxic. In a 2015 study comparing TMAO levels in patients with CKD ($n$ = 521) to healthy patients ($n$ = 3166), median TMAO levels among CKD patients were significantly higher ($P$ < 0.001)[70]. Similarly, a 2019 case-control study comparing patients with NAFLD ($n$ = 34) to those without ($n$ = 14) showed that TMAO has a role in aggravating liver steatosis[71]. Lastly, certain species in the gut microbiota produce short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate and diffuse through gut mucosa, which can disrupt the integrity of the intestinal barrier. In the bloodstream, SCFAs can cause systemic inflammation, the common pathogenic link between NAFLD and CKD[45].

**Genetic links between NAFLD and CKD:** Two gene variants associated with both CKD and NAFLD are the G allele of the *patatin-like phospholipase domain-containing* (

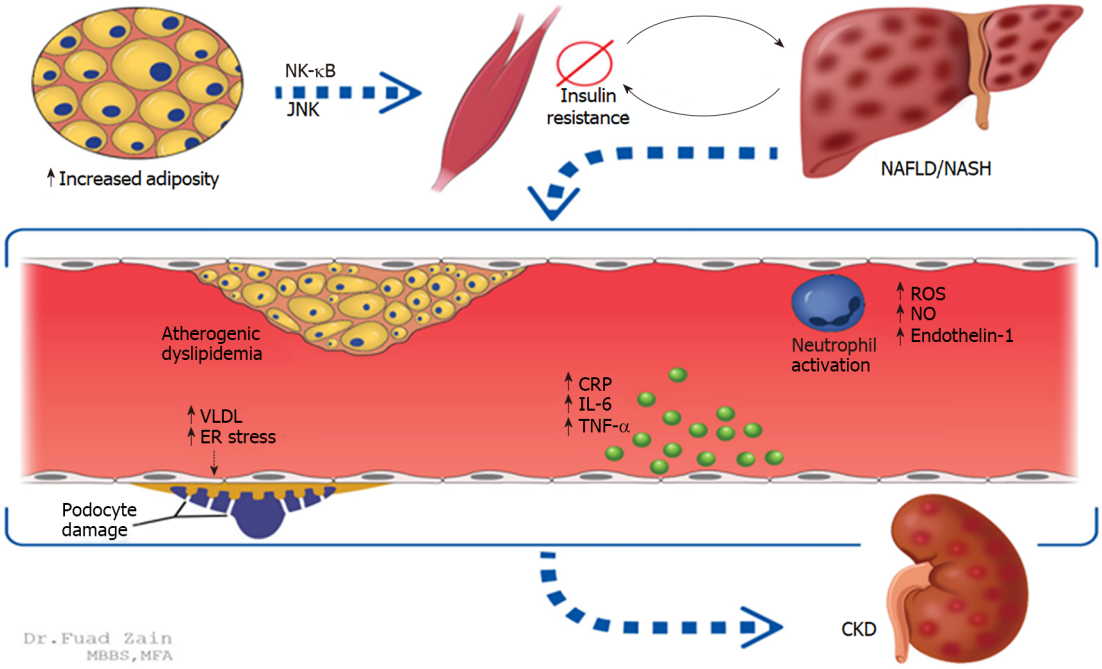


**Figure 1 Two established mechanisms between non-alcoholic fatty liver disease and the development of chronic kidney disease are increased adiposity and insulin resistance.** NF-κB: Nuclear factor-κB; JNK: Jun N-terminal kinases; NAFLD/NASH: Non-alcoholic fatty liver disease/Non-alcoholic steatohepatitis; ROS: Reactive oxygen species; NO: Nitric oxide; CRP: C-reactive protein; IL-6: Interleukin-6; VLDL: Very low-density lipoprotein; TNF-α: Tumor necrosis factor alpha; CKD: Chronic kidney disease

*PNPLA3)* gene and the T allele of the *transmembrane 6 superfamily member 2* (*TM6SF2*) gene. The G allele in the rs738409 polymorphism of the *PNPLA3* gene has been shown to play a major role in the progression of NASH[72,73]. Patients with the G allele also have been shown to have lower eGFR, increased incidence of microalbuminuria, and increased prevalence of CKD, regardless of NAFLD/NASH status[74,75]. The patient population that was found to have the highest risk of CKD and NAFLD in a 2015 study were patients who carried the G allele of the *PNPLA3*; furthermore, these patients were not obese, which is an important risk factor for CKD[75]. Another study showed that Chinese patients with normal alanine aminotransferase levels who carried the rs738409 polymorphism in the *PNPLA3* gene were at risk for early glomerular and tubular damage, which could explain why these patients develop CKD even in the absence of well-known risk factors, such as obesity or diabetes[76]. In postmenopausal women with T2DM, having the G/G allele leads to a higher prevalence of CKD, regardless of NAFLD status, further supporting the argument that this polymorphism may be an independent predictor for CKD[77]. Patients who are found to have the G/G allele in the polymorphism rs738409 should have close monitoring for the development of NAFLD as well as renal dysfunction, even in normal-weight patients[75]. On the other hand, the rs58542926 polymorphism on the *TM6SF2* gene, also known as the T allele of the *TMS6F2* gene, has been associated with the development of NAFLD[78] but has also been associated with a higher eGFR and lower prevalence of microalbuminuria[74]. Thus, this specific polymorphism in *TM6SF2* may be nephroprotective in patients with NAFLD.

### *Identifying NAFLD patients at risk for progression of CKD*

**Role of non-invasive fibrosis scoring systems:** Non-invasive scoring systems are utilized in assessing the severity of various chronic liver diseases. However, they have also been shown in several studies to be useful in predicting CKD in patients with NAFLD (Table 3). Incremental increases in the fatty liver index are an independent risk factor for developing CKD in a 10-year prospective analysis of 6238 adults (age 40-69 years) without CKD at baseline[23]. In another study of 11376 Taiwanese subjects, the NFS was negatively correlated with eGFR[6]. Multiple studies show that patients who have an intermediate and high-risk category of fibrosis-4 index (FIB-4)-index and NFS are at an increased risk of CKD[79,80], while a 2019 cross-sectional study of 11836

**Table 3 Summary of studies assessing non-invasive scoring systems for advanced fibrosis to assess risk for chronic kidney disease in patients with nonalcoholic fatty liver disease**

| Ref. | Year | *n* | Scoring system(s) assessed | Results |
|---|---|---|---|---|
| Ciardullo *et al*[82] | 2020 | 2770 | APRI, FIB-4, FLI, NFS | NAFLD-related fibrosis as measured with FIB-4 associated with CKD ($P < 0.01$) |
| Hsieh *et al*[6] | 2020 | 11376 | NFS | Higher NFS associated with impaired eGFR ($P < 0.0001$) |
| Choi *et al*[81] | 2019 | 11836 | APRI, BARD, FIB-4, FLI | FIB-4 ($P = 0.0258$) most precise in predicting kidney dysfunction |
| Önnerhag *et al*[79] | 2019 | 144 | APRI, BARD, NFS, FIB-4 | High-risk NFS ($P < 0.001$), FIB-4 ($P < 0.001$), APRI ($P = 0.008$) predict CKD |
| Wijarnpreecha *et al*[80] | 2018 | 4142 | APRI, BARD, NFS, FIB-4 | High/intermediate probability of liver fibrosis on NFS (AUC = 0.75) and FIB-4 (AUC = 0.77) independently predict CKD |
| Huh *et al*[23] | 2017 | 6238 | FLI | NAFLD cut-off for NAFLD is an independent RF for CKD ( $P < 0.0001$) |

NFS: Nonalcoholic fatty liver disease fibrosis score; FIB-4: Fibrosis-4 index; APRI: Aspartate aminotransferase to platelet ratio index; FLI: Fatty liver index; CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; AUC: Area under the curve; RF: Risk factor; BARP: Biologically-oriented Alveolar Ridge Preservation; NAFLD: Nonalcoholic fatty liver disease.

patients showed that FIB-4 is the most precise tool when estimating renal dysfunction attributable to NAFLD (area under the curve = 0.6227, 95%CI: 0.5929-0.6526, $P = 0.0258$) after adjusting for various demographic and clinical variables[81]. FIB-4 is the most superior predictor in other studies as well[80,82]. In summary, patients with NAFLD-related fibrosis are at increased risk for CKD, and these patients should undergo proper surveillance *via* non-invasive fibrosis scoring systems and/or advanced imaging techniques (*i.e.* Fibroscan, TE) (Figure 3).

**Cystatin C:** Serum creatinine, a widely used biomarker in assessing renal function, is inaccurate in determining GFR in patients with cirrhosis[83]. This is due to muscle wasting that occurs in cirrhosis, thus leading to diminished creatinine formation, increased tubular secretion of creatinine, and impaired assay interpretation caused by elevated bilirubin[83]. Alternatively, the measurement of cystatin C does not have the same limitations as serum creatinine due to its low molecular weight and because it does not require adjustment for gender, mass, or bilirubin level[84]. A combination of serum creatinine and cystatin C is more accurate in determining GFR than serum creatinine alone[85]. However, serum creatinine alone is superior for patients without cirrhosis[85]. Measurement of cystatin C in addition to serum creatinine may have utility for accurately assessing renal function in transplant candidates and for monitoring the development of CKD in patients with NASH cirrhosis. Although the cost of measuring eGFR using Cystatin C in addition to serum creatinine is higher, the burden of over-diagnosing CKD in patients with cirrhosis is lessened, which may lead to an overall reduction in unnecessary medical expenses for patients with cirrhosis who truly have CKD[86].

**Alkaline phosphatase and GGT:** In diabetic patients with NAFLD, serum alkaline phosphatase (ALP), a NAFLD-associated marker when elevate, was also significantly associated with impaired renal function[87,88]. Interestingly, ALP is associated with the release of proinflammatory cytokines from the liver that are known to disrupt the glomerular endothelial glycocalyx, leading to albuminuria, which may explain why ALP is a potential surveillance marker in patients with NAFLD who are at risk for developing CKD[89]. Furthermore, elevated serum GGT is associated with an increased risk of CKD[24,90,91]. GGT is associated with increased inflammatory markers and insulin resistance, both of which play central roles in patients with NAFLD who develop CKD[24,92]. However, elevated GGT may not be an accurate CKD parameter in Caucasian men, as GGT is confounded by BMI, lifestyle factors, and lipids, as noted in a 2017 study[25]. Therefore, elevated GGT in Caucasian men with NAFLD should be interpreted with caution when monitoring for CKD. Of importance, NAFLD was diagnosed by elevated GGT levels (in addition to ultrasound in only one study[91]; therefore, these findings may not apply to patients diagnosed by more

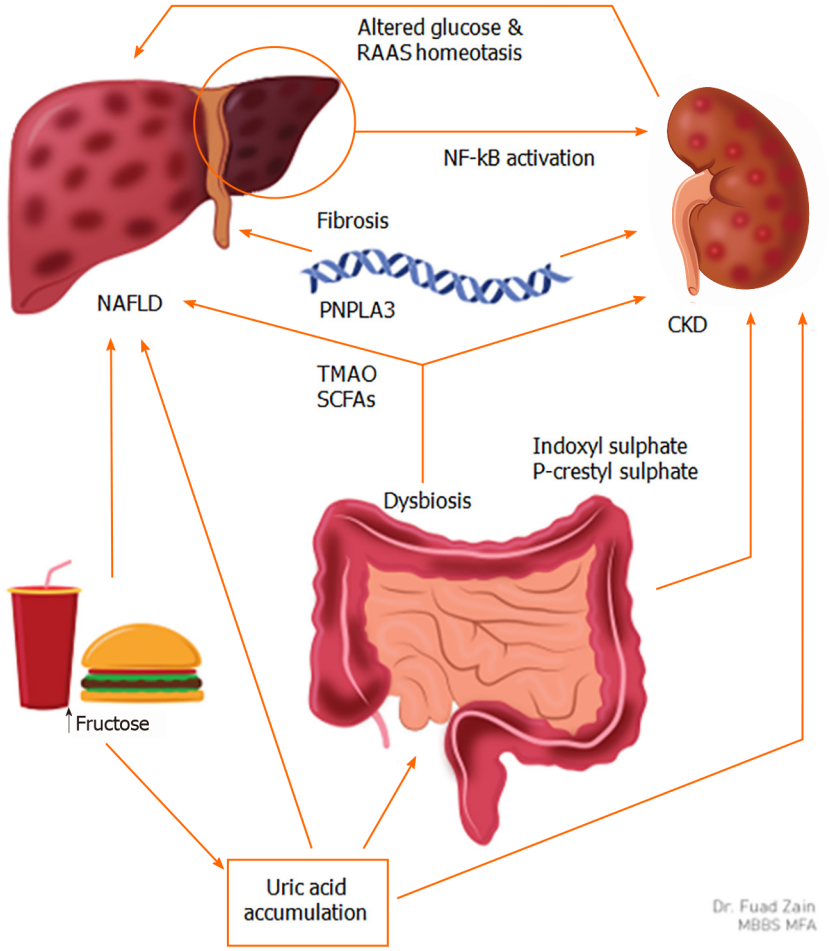


**Figure 2 Fructose consumption and uric acid accumulation play a key role in patients with non-alcoholic fatty liver disease who develop chronic kidney disease.** TMAO: Trimethylamine N-oxide; SCFAs: Short-chain fatty acids; RAAS: Renin-angiotensin-aldosterone system; PNPLA3: Patatin-like phospholipase domain-containing protein 3; NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; NF-κB: Nuclear factor-κB.

invasive parameters (*i.e.* liver biopsy).

*Managing the progression of CKD*

**Surveillance of comorbidities**: In general, we recommend patients with diabetes and NAFLD undergo frequent surveillance for underlying kidney dysfunction, more so than patients with diabetes only. Monitoring thyrotropin and thyroid hormone levels may have clinical utility when evaluating the risk of developing CKD in patients with NAFLD; however, future studies are needed to specifically address the risk of CKD in patients with NAFLD and hypothyroidism (Table 4).

### Body weight

**Waist-to-hip ratio:** Few studies have evaluated the impact of weight loss on the progression of CKD in patients with NAFLD. Recent studies have shown that a decrease in the waist-to-hip ratio (WHR) in patients with NAFLD decreases the risk of CKD development[43]. Serial monitoring of WHR may be beneficial in identifying patients with NAFLD at risk for CKD. A drawback to this finding is that a reduction in WHR does not differentiate between a reduction in visceral fat *vs* subcutaneous fat. Studies have shown that visceral fat, but not subcutaneous fat, is the key driver in NAFLD pathogenesis *via* increased insulin resistance[93]. However, with regards to reducing the risk of CKD, the significance of reducing visceral *vs* subcutaneous fat is not well-studied.

**Weight loss:** Data from a post-hoc analysis of a clinical trial involving 261 patients with NAFLD showed a statistically significant relationship between reduction in body weight and changes in eGFR (when calculated by CKD-Epidemiology collaboration and modification of diet in renal disease equations), even after adjusting for

**Table 4 Summary of Interventions for patients with nonalcoholic fatty liver disease and chronic kidney disease**

| Intervention | Ref. | Year | *n* | Findings | Recommendation |
|---|---|---|---|---|---|
| Decreasing WHR | Chon *et al*[43]. 12-yr prospective cohort | 2020 | 6137 | A decrease in the WHR of more than 5% in patients with NAFLD leads to a significantly reduced risk of CKD development, even in non-obese patients | Serial Monitoring WHR may be beneficial in identifying patients with NAFLD at risk of developing CKD and reduction can ameliorate the progression |
| Weight loss | Vilar-Gomez *et al*[94]. Post-hoc analysis | 2017 | 261 | Improvement in liver histology due to weight loss linked to improved renal outcomes, even after adjusting for medication profile, diabetes, and hypertension | Advocate for weight loss |
| SGLT2 Inhibitors | Shimizu *et al*[96]. RCT | 2019 | 57 | SGLT inhibitor (Dapagliflozin) improved liver steatosis in patients with T2DM and NAFLD and attenuates liver fibrosis in patients with NAFLD-related advanced fibrosis | Although data is not sufficient, consider using SGLT2 inhibitors in T2DM patients with NAFLD and CKD |
| | Perkovic *et al*[95]. CREDENCE trial | 2019 | 4401 | SGLT2 inhibitor (Canagliflozin) decreased the risk of renal failure in patients with T2DM and CKD | |
| GLP-1 | Armstrong *et al*[100]. LEAN trial | 2016 | 52 | Liraglutide led to weight loss, glycemic control, and histological resolution of NASH | GLP-1's in NASH is considered effective in improving components of MetS, however, long-term studies are needed to determine NASH-related outcomes |
| | Tuttle *et al*[101]. AWARD-7 trial | 2018 | 577 | Once-weekly dulaglutide is associated with reduced decline in eGFR, while being as effective as insulin in achieving glycemic control | GLP-1 is a safe option for patients with CKD and is associated with slower progression of CKD |
| Coenzyme Q10 | Farhangi *et al*[109] and Farsi *et al*[110]. RCT | 2014[109] and 2016[110] | 44[109] and 41[110] | 100 mg of oral CoQ10/d improve biochemical variables of NAFLD after 4 wk[109] and 12 wk[110] of treatment | Due to lack of data in patients with both NAFLD and CKD, the benefit of CoQ10 supplementation is unknown; however, in separate trials with regards to both NAFLD and CKD, CoQ10 supplementation is beneficial |
| | Yeung *et al*[111]. RCT | 2015 | 15 | Oral CoQ10 supplementation in patients with CKD showed significant improvement in serum creatinine when compared to placebo | |

WHR: Waist-to-hip ratio; NAFLD: Nonalcoholic fatty liver disease; CKD: Chronic kidney disease; SGLT2: Sodium-glucose co-transporter-2; RCT: Randomized controlled trial; T2DM: Type 2 Diabetes Mellitus; GLP-1: Glucagon-like peptide receptor agonist; NASH: Non-alcoholic steatohepatitis.

medication profile, diabetes, and hypertension[94]. Additionally, patients with improvement in liver histology due to lifestyle modifications such as weight loss were linked with significantly improved renal outcomes[94]**.** Overall, patients with NAFLD who had more than 5% weight loss and/or more than a 5% reduction in WHR had improved renal outcomes.

**Sodium-glucose cotransporter type-2 inhibitors:** In patients with T2DM, sodium-glucose cotransporter type-2 (SGLT2) inhibitors have an established role in improving glycemic control, weight loss, cardiovascular outcomes, and lowering serum uric acid levels. In patients with type 2 diabetes, the landmark CREDENCE trial showed that patients treated with an SGLT2 inhibitor (*i.e.*, canagliflozin) were shown to have improved outcomes related to CKD[95]. Furthermore, recent evidence has shown that SGLT2 inhibitors can also improve NAFLD progression as determined by TE[96] and biomarkers in NAFLD (*i.e.*, liver enzymes)[96,97]. As SGLT2 inhibitors decrease serum uric acid levels, this may also contribute to this class's positive effects on both diseases. In addition to facilitating glucosuria, SGLT2 inhibitors are thought to decrease inflammation and reactive oxygen species formation[98], which is key in the pathogenesis of NAFLD and NASH[99].

**Glucagon-like peptide 1 receptor agonists:** Among its multiple mechanisms of action, glucagon-like peptide 1 **(**GLP-1)'s aid in increasing insulin secretion, delaying gastric

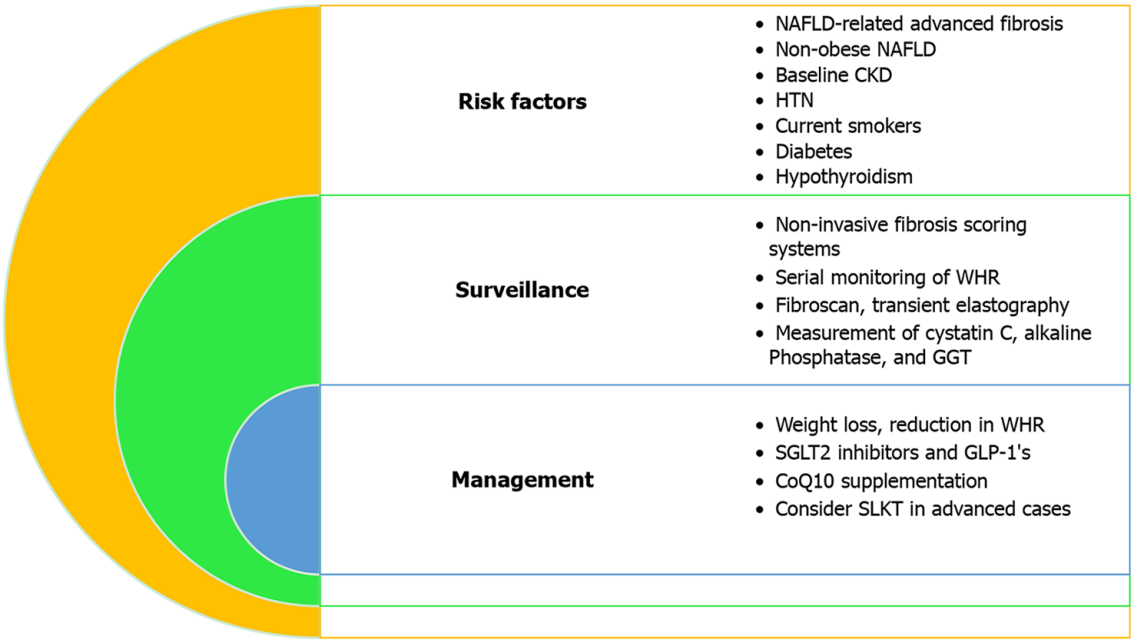


**Figure 3 Identifying and managing non-alcoholic fatty liver disease patients who are at risk for developing chronic kidney disease.**
NAFLD: Non-alcoholic fatty liver disease; CKD: Chronic kidney disease; HTN: Hypertension; WHR: Waist-to-Hip ratio; GGT: Gamma-glutamyl transferase; T2DM: Type 2 diabetes mellitus; SLKT: Simultaneous liver-kidney transplantation; SGLT2: Sodium-glucose cotransporter type-2; GLP-1: Glucagon-like peptide 1.

emptying, and decreasing appetite, all of which can lead to improved glycemic control and weight loss. Additionally, a possible anti-inflammatory mechanism makes GLP-1's an attractive agent in NAFLD and NASH. For instance, GLP-1 Liraglutide, when compared to placebo, led to histological resolution of NASH; however, larger studies are still needed[100]. In CKD, GLP-1's are shown to be nephroprotective, which could be due to GLP-1's ability to lower blood pressure in addition to the aforementioned mechanisms[101,102]. GLP-1's and SGLT2 inhibitors exhibit cardioprotective effects, and as discussed previously, patients with NAFLD and CKD are at high risk for CV events. Therefore, the use of these agents is recommended in patients with NAFLD and CKD. However, while there is landmark data to support the use of GLP-1's and SGLT2 inhibitors to prevent CV events in patients with established CVD, data on primary prevention in patients with NAFLD and CKD is lacking[103,104]. Regardless, in patients with T2DM, CKD, and NAFLD, SGLT2 inhibitors or GLP-1's are highly recommended not only for glycemic control but for the cardio-, hepato-, and nephro-protective effects as well.

**Coenzyme Q10:** Coenzyme Q10 (CoQ10) is produced endogenously and has antioxidant and anti-inflammatory effects[105]. CoQ10 also serves as an electron carrier in cellular respiration and a cofactor in pyrimidine synthesis for DNA repair and replication, among other important roles. Patients with NAFLD, CKD, and/or CVD have been reported to have CoQ10 deficiency[106]. A majority of endogenous CoQ10 is produced in the liver, and patients with NAFLD had diminished CoQ10 production[106,107]. CoQ10 deficiency will lead to oxidative stress, which plays a key pathogenic factor in NAFLD[108]. Results from separate trials assessing oral CoQ10 supplementation in patients with NAFLD and CKD are summarized in Table 4. Briefly, CoQ10 has been shown to improve NAFLD parameters and CKD parameters in separate trials[109-111]. Specific findings are summarized in Table 4. CoQ10 has positive effects on the progression of CVD as well, which is notable because patients with CKD and NAFLD are at risk for cardiovascular events[112,113]. Supplementation may be beneficial in patients with NAFLD who have CKD, but clinical data in this population is lacking.

### *Experimental interventions*

**Thiazolidinediones:** Thiazolidinediones are agonists of peroxisome proliferator-activated receptors (PPARs), and they play a physiologic role in metabolism and cellular differentiation. PPARs have proven clinical utility in diseases such as hyperlipidemia (PPARα) and T2DM (PPARγ)[114]. Because CKD is a manifestation of a

metabolic and/or inflammatory process, the use of PPAR agonists has been studied in patients with CKD. Specifically, pioglitazone, a PPARγ agonist, has been shown to improve cardiovascular outcomes in patients with CKD and diabetes[115]. Several RCTs have shown the beneficial effects that pioglitazone has on histopathology and metabolic function in patients with NASH[116-120]. Pioglitazone has been endorsed as a pharmacological agent in biopsy-proven NASH by the American Association for the Study of Liver Diseases[121]. Rosiglitazone has been shown to improve histological components of NASH through increasing insulin sensitivity[122] while also improving liver function[123] in a separate study, although both studies did not show improvements in liver fibrosis[122,123]. Interestingly, an extension trial showed that rosiglitazone was only beneficial in the first year of treatment, without substantial benefit noted with longer use[124]. However, Rosiglitazone is not available in most countries and its use is limited in the United States due to data concerning for increased coronary events. The most widely studied PPAR agonist, Pioglitazone has shown favorable outcomes in patients with CKD and patients with NAFLD, but data assessing the efficacy in patients with both CKD and NAFLD is lacking[114].

**Vitamin D:** Vitamin D deficiency is associated with increased severity of NAFLD[125] and is also associated with CKD[126]. These findings may be explained by the physiology of vitamin D activation, which requires hydroxylation by both the kidney and liver, and therefore the presence of CKD and NAFLD inevitably leads to vitamin D resistance[58]. Furthermore, experimental models have demonstrated the role hypovitaminosis D plays in the pathogenesis of both CKD and NAFLD[58]. In patients with CKD, therapeutic implications of higher vitamin D supplementation showed an ability to correct hypovitaminosis D[127], but a meta-analysis yielded a higher incidence of hypercalcemia[128]. In patients with NAFLD, vitamin D supplementation did not correct hypovitaminosis D[129], however, trials are underway for assessing the use of Vitamin D supplementation in CKD and NAFLD/NASH[130-132] (NCT00893451, NCT01623024, and NCT02098317, www.clinicaltrials.gov).

**Probiotics**: In rodent models, fecal microbiota transplantation[133], antibiotics in fructose-fed models[134] reduced NAFLD severity, whereas specific probiotics (*Lactobacillaceae* or *Bifidobacteriales*) alleviated proteinuria and reduced systemic inflammation in rodents with CKD. While much of this data is based on studies from animal models, human trials are needed to further evaluate the therapeutic implications of the gut-liver-kidney axis.

### *LT for patients with NAFLD/NASH and CKD*

In recent decades, NASH has become more prevalent and will become the most common indication for LT[135]. Patients with NASH have a higher incidence of CKD compared with other etiologies, and therefore, NASH is rapidly growing as a cause for not only LT[136,137] but also simultaneous liver-kidney transplantation (SLKT) in the United States given serum creatinine and dialysis status are important components of the model for end-stage liver disease (MELD) score[138]. Considering the increased incidence of renal dysfunction at LT due to prioritization based on the MELD allocation system in the United States, SLKT rates climbed from 2.7% of all LT in 2000 to 9.3% in 2016[138-140]. NASH is currently the leading and most rapidly growing indication for SLKT in the United States[138,140] with a 200% increase for SLKT from 2002 to 2010[138]. Patients with NASH have a high probability to undergo SLKT rather than LT alone since they are highly incident for CKD for a prolonged duration, which can fulfill criteria for SLKT (patients with CKD: GFR ≤ 60 mL/min for ≥ 3 mo with recent GFR ≤ 30 mL/min or on hemodialysis, patients with AKI: Dialysis for > 6 wk GFR ≤ 25 for > 6 wk)[141].

Patients with NASH were independently associated with a higher risk of CKD or advanced kidney damage after LT compared with those without NASH[142-144]. In general, renal dysfunction after LT is affected not only by immunosuppressant medications, especially calcineurin inhibitors, pre-LT kidney dysfunction, but also persistent or de novo metabolic co-morbidities such as hypertension, diabetes, and obesity - all of which are highly likely in NASH patients undergoing LT[142,145]. Special attention to the recognition of CKD is needed for patients with NASH patients when deciding LT *vs* SLKT. Controlling for metabolic complications and avoiding or keeping a low dose of calcineurin inhibitors as much as possible seems to be crucially important to reduce the risk of incident CKD, and risk of progression of CKD after liver transplant in NASH patients.

## CONCLUSION

Despite the breadth of research, minimal guideline-based management of patients with both NAFLD and CKD is available. However, important pathogenic links and shared risk factors between NAFLD and CKD underscore the importance of earlier surveillance and strict control of shared metabolic risk factors. Although preventative strategies for CKD in NAFLD are limited, treatment directed specifically for NASH in the future will hopefully ameliorate the progression of renal dysfunction in affected patients. There is a plethora of clinical trials underway, and if these drugs show safety and efficacy in improving NASH, they may translate into improving renal function[146]. Specific interventions for preventing CKD progression using SGLT2 inhibitors, PPAR agonists, SAM, XO inhibitors, and Vitamin D have been tried but need further confirmation. Progression from NAFLD to NAFLD-related advanced fibrosis is linked to an increased risk of CKD, and earlier intervention in those with renal dysfunction is warranted. Genetic links between NAFLD and CKD have also been proposed, specifically in the G allele of *PNPLA3* and the T allele of *TM6SF2*, and future studies targeting patients with such genetic profiles to prevent progression to CKD is needed.

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*REVIEW*

# Repurposing metformin for the treatment of gastrointestinal cancer

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## Abstract

Diabetes mellitus type 2 and cancer share many risk factors. The pleiotropic insulin-dependent and insulin-independent effects of metformin might inhibit pathways that are frequently amplified in neoplastic tissue. Particularly, modulation of inflammation, metabolism, and cell cycle arrest are potential therapeutic cancer targets utilized by metformin to boost the anti-cancer effects of chemotherapy. Studies *in vitro* and *in vivo* models have demonstrated the potential of metformin as a chemo- and radiosensitizer, besides its chemopreventive and direct therapeutic activity in digestive system (DS) tumors. Hence, these aspects have been considered in many cancer clinical trials. Case-control and cohort studies and associated meta-analyses have evaluated DS cancer risk and metformin usage, especially in colorectal cancer, pancreatic cancer, and hepatocellular carcinoma. Most clinical studies have demonstrated the protective role of metformin in the risk for DS cancers and survival rates. On the other hand, the ability of metformin to enhance the actions of chemotherapy for gastric and biliary cancers is yet to be investigated. This article reviews the current findings on the anti-cancer mechanisms of metformin and its apparatus from pre-clinical and ongoing studies in DS malignancies.

**Key Words:** Antidiabetic treatments; Gastrointestinal tumors; Therapeutic target



**Core Tip:** Modulation of cell function into the neoplastic and around the microenvironment tissue are possible cancer targets utilized by metformin to raise

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chemotherapy's anti-tumor outcomes. Herein we review the studies that have demonstrated the likelihood of metformin as chemo and radiosensitizer, in addition to its chemopre-ventive and direct therapeutic activity in gastrointestinal tumors.

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## INTRODUCTION

Diabetes mellitus type 2 (DM2) and cancer share several risk factors[1]. Notably, obesity and metabolic syndrome, with their inherent biological connections, such as hyperinsulinemia[2] and chronic inflammation[3]. Furthermore, some antihyperglycemic medications (*e.g.*, sulfonylureas and insulin) used for the treatment of DM2 may increase cancer risk[4]. Particularly, central obesity, physical inactivity, and perhaps a low dietetic polyunsaturated fat to saturated fat ratio are major risk factors for insulin resistance and hyperinsulinemia and seem to be related to cancer risk[5]. All of them have been recognized as proposed gears holding those relationships[6-9]. Epidemiologic studies and meta-analyses have suggested that patients with DM2 have a higher incidence and mortality from malignancies[10,11], including digestive system (DS) cancers[12-15].

Metformin is a well-known oral hypoglycemic drug that belongs to the biguanide class and has been used to treat DM2 for almost a century[16]. Importantly, those patients with DM2 with long-term use of metformin have a decreased tumor incidence and lower cancer-associated mortality[17-21]. Furthermore, recent research indicates that metformin can have direct anti-cancer activity against many tumor cells, including tumor stem cells[22,23], therefore, carrying out pleiotropic effects in both the cancer cell and the neoplastic microenvironment[24]. Their potential mechanisms are insulin-dependent [*via* insulin growth factor (IGF) receptor, phosphatidyl inositol 3 kinase (PI3K), and Akt/mammalian target of rapamycin (mTOR)][25,26] and insulin-independent [*via* adenosine kinase monophosphate (AMPK), tuberous sclerosis complex (TSC), and mTOR][27,28]. Moreover, it promotes antitumor immunity-related metabolic checkpoints in T-cells, cancer cells, as well as associated with immunosuppressive cells of the tumor milieu[29]. Furthermore, it might interfere with the gut microbiota and have systemic impacts on body metabolism[30,31]. This article aims to review the rationale of metformin as a drug that might be repurposed for DS cancer treatment.

## MECHANISM OF ACTION OF METFORMIN AS AN ANTI-CANCER AGENT

Two potential mechanisms for the antineoplastic action of metformin have been suggested (Figure 1). First, metformin can directly activate AMPK, resulting in inhibition of downstream Akt/mTOR signaling and consequent suppression of cell proliferation[32,33]. Second, metformin-induced reductions in circulating insulin and IGF concentrations may reduce activation of the IGF receptor signaling axis, resulting in decreased growth promotion and mitogenesis[2,34]. Hence, the anti-cancer effects of metformin are mediated through a systemic improvement in the metabolic milieu or directly on tumor cells[35].

The noticeable intracellular metabolic change caused by metformin is the decreased accumulation of glycolytic intermediates and a coordinated decrease in tricarboxylic acid (TCA) cycle intermediates[36,37]. Moreover, the activation of AMPK reduces fatty acid synthase (FAS) gene expression in the synthesis of fatty acids[32]. Furthermore, metformin offers other direct anti-tumor effects by (1) decreasing specific protein (Sp) transcription factors and Sp-related oncogenic proteins[38,39]; (2) decreasing AMPK-dependent c-Myc oncogene; (3) increasing other miRNAs, such as mir33a[40]; (4) increasing other miRNAs, such as miR-26a[41]; (5) reducing endogenous reactive oxygen species and associated DNA damage[42]; (6) reducing Sonic hedgehog

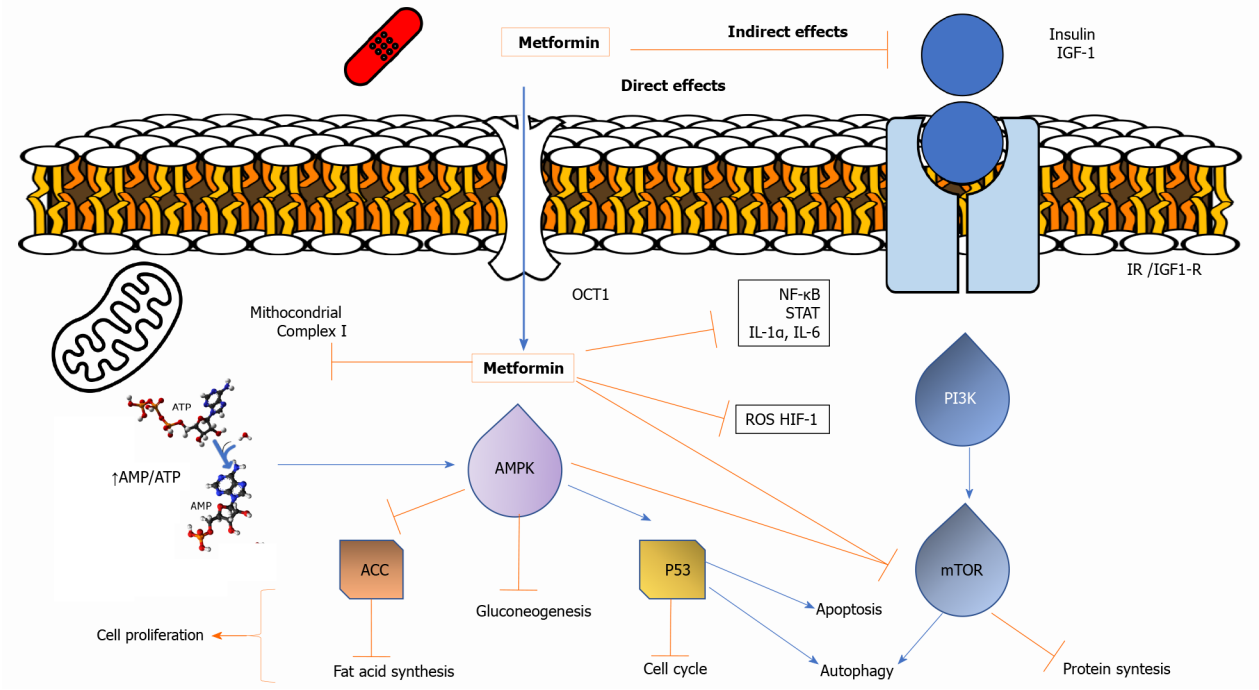


**Figure 1 Overview of cellular mechanisms of metformin in cancer.** Metformin inhibits mitochondria complex I, stimulates the adenosine monophosphate-activated protein kinase signaling pathway, and/or inhibits the insulin signaling pathway. Blue lines represent activated pathways while red lines represent inhibitory pathways. AMPK: Adenosine monophosphate-activated protein kinase; ACC: Acetyl-CoA carboxylase; HIF-1α: Hypoxia-inducible factor-1 alpha; IGF: Insulin growth factor; IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IR: Insulin receptor; IL-1: Interleukin 1; IL-6: Interleukin-6; NF-κB: Nuclear factor kappa; OCT1: Organic cation transporter 1; ROS: Reactive oxygen species; STAT: Signal transducer and activator of transcription; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; PI3K: Phosphoinositide 3-kinase; mTOR: Mechanistic target of rapamycin.

expression[43]; (7) reducing expression of angiogenic factor CCN1, which inhibits invasion induced by the stromal cell-derived factor-1 and reducing levels of type 4 chemokine receptor[44]; and (8) inhibiting Rac1 GTPase activity[45]. Finally, metformin might interfere with the gut microbiota[30,31], as well as interfere with the balance between T-cells and associated immunosuppressive cells in the tumor milieu[29].

### *Insulin-dependent or indirect effects*

A central signal transduction pathway involved in cancer is the PI3K/Akt/mTOR pathway, which, when hyperactivated, leads to deregulation of survival and cell growth[28,46,47]. IGF-1 is a more potent mitogen than insulin and, like insulin, binds to its particular growth factor receptor and stimulates cell growth and anti-apoptotic activity *via* MAPK/ERK or Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling [2,34]. In addition, IGF-1 inhibits PTEN, a phosphatase that deactivates PI3K/Akt/mTOR[2]. The indirect mechanisms of metformin action include inhibition of hepatic gluconeogenesis and stimulation of peripheral glucose absorption, which ultimately lead to decreased blood glucose and insulin levels. Thus, the most apparent mechanism of insulin-dependent metformin involves decreasing insulin levels, which reduces insulin binding to the insulin receptor (IR), inhibiting tumor growth[48]. A reduction of insulin/IGF-1 levels is, at least in part, involved in the antiproliferative activity of metformin[49]. Additionally, metformin downregulates IGF-R and IR by decreasing the promoter activity of receptor genes with subsequent Akt/mTOR and MAPK/ERK signaling inhibition[50,51].

### *Insulin-independent or direct effects*

Metformin activates AMPK by inhibiting mitochondrial complex I, which leads to impaired mitochondrial function, decreased adenosine triphosphate synthesis, increased adenosine monophosphate, and subsequent phosphorylation and activation by LKB1[52]. Activated AMPK then phosphorylates TSC2, which negatively regulates mTOR activity[53]. Activation of LKB1 and AMPK, AMPK-induced stabilization of TSC1-TSC2 (inhibitor of Rheb, an mTORC1 activator), and activation of the tumor suppressor p53[54]. Moreover, independent of AMPK, metformin impedes mTORC1 by raising p53-dependent expression of REDD1 and repressing Rags[55]. Metformin

also retards transformation by inhibiting mediators of the inflammatory response, including transcription factors (nuclear factor kappa, Signal transducer and activator of transcription 3, and Forkhead box O signaling), and downregulating Lin28B, most Let-7 miRNA family members, and inflammatory molecules [interleukin (IL)-1α, IL-1β, IL-6, and vascular endothelial growth factor (VEGF)][56].

Metformin has other AMPK-mediated actions that may be implicated in cancer. Through the activation of AMPK, metformin causes the suppression of FAS gene expression, which is involved in the synthesis of fatty acids, resulting in reduced lipogenesis, increased fatty acid oxidation, and decreased cell proliferation[32,57]. The activation of AMPK also modulates cyclin D1 (cell cycle protein), p21 and p27 (cyclin-dependent protein kinase), which further contribute to its anti-cancer effects[55,58]. Interestingly, metformin may act as a chemosensitizer, for example, increasing the 5-fluorouracil (5-FU) and paclitaxel sensitivities of cancer cell lines[59,60]. The ability of metformin to disconnect the electron transport chain by inhibiting complex I (NADH dehydrogenase) strongly induces cell death when glucose is limited. Metformin also reduces the hypoxic activation of hypoxia inducing factor (HIF-1), suggesting that the effects of metformin are increased in hypoglycemic and hypoxic conditions[61].

### *Other mechanisms*

As a drug that controls metabolism, metformin promotes a coordinated decrease of TCA cycle intermediates, including succinate, fumarate, malate, citrate, and α-ketoglutarate[36,37]. The dependency of neoplastic cells on glutamine metabolism has been shown to be reprogrammed by the Kras oncogenic pathway through a single pathway involving serum glutamic-oxaloacetic transaminase, which maintains the cellular redox states essential to mitochondria and offers innovative therapeutic targets in combination with metformin[62].

Metformin can exert antitumor activity by increasing CD8+ T-cells[63,64]. It might inhibit apoptosis of CD8+ tumor infiltrating lymphocytes and prevent immune exhaustion[63,65]. Furthermore, metformin might adjust the expression profile of immune checkpoints[66], such as programmed death ligand 1, in the context of the neoplasm[37], thereby suggesting that a combination of metformin might have the potential to enhance the strength of cancer immunotherapy[63].

There is evidence that epigenomic modifications by metformin may contribute to its anti-cancer properties[67]. Metformin might regulate the activity of numerous epigenetic modifying enzymes, principally by modulating the activation of AMPK. Activated AMPK can phosphorylate several substrates, comprising epigenetic enzymes, such as histone acetyltransferases (HATs), class II histone deacetylases (HDACs) and DNA methyltransferases (DNMTs), usually resulting in their inhibition; however, HAT1 activity may be increased. Metformin has also been related to the diminished expression of various histone methyltransferases[68], enhancing the activity of the class III HDAC SIRT1 and minimizing the influence of DNMT inhibitors[69,70].

## METFORMIN STUDIES IN DIGESTIVE SYSTEM MALIGNANCIES

### *Metformin in colorectal cancer*

**Cell lines and animal models:** Metformin promotes cell cycle arrest in the G0/G1 phase in colorectal cancer (CRC) cell lines. It also decreases the expression of c-Myc and causes down-regulation of IGF-1R[71]. Consequently, up-regulation of the adenosine A1 receptor induces apoptosis[72]. Additionally, it was shown that metformin enhances the activity of the *Sprouty2* gene, which suppresses colon cancer growth[73].

The combination of metformin with 5-FU was investigated on the SW620 CRC cell line and on patients with DM2. The study showed that metformin plus 5-FU treatment significantly inhibited the proliferation of SW620 cells compared with that in monotherapy. Additionally, the examination of 86 CRC tissue samples obtained from patients with DM2 revealed that treatment with metformin decreased the proportion of poorly differentiated tumors[74]. Moreover, a synergistic effect of 5-FU and metformin was observed in a 5-FU resistant cell line[74] and metformin radiosensitizer CRC cells, with reduced survival of ionization-resistant cells[75]. Consistently, the association of oxaliplatin, 5-FU and metformin also demonstrated a superior anti-tumor activity in chemoresistant HT-29, and HCT-116 cells compared to that with the drugs separately[76].

In 1977, it was firstly reported that phenformin inhibits metabolic immunosuppression in rats[77]. Since then, several reports have demonstrated that metformin has both chemopreventive and therapeutic activities in animal models of CRC. For instance, metformin treated ApcMin/+ mice showed significantly smaller polyps[78], and carcinogen-induced animal models that received metformin had a reduction of aberrant crypt foci[79], indicating the chemopreventive effect of metformin. Moreover, the association of metformin with D3 vitamin demonstrated a chemopreventive effect against 1,2-dimethylhydrazine (DMH)-induced CRC in rats and DMH-dextran sodium sulfate-induced colitis-associated CRC in mice[80]. On the other hand, treatment with metformin, 5-FU and oxaliplatin demonstrated superior antiproliferative effects in SCID mice bearing CRC[76]. In avatar models, metformin suppressed the tumor growth in the patient-derived xenografts by 50%[81]. In the same study, when metformin was combined with 5-FU, the tumor growth was inhibited up to 85%[81].

### Clinical use of metformin

**Metformin and CRC risk:** As shown in Table 1 many case-control and cohort studies and associated meta-analyses have evaluated DS cancer risk and metformin use. Specifically, a decreased risk of CRC was found in the majority of studies[82-86], but no association or an increased risk of CRC was found in some of them[87-91]. Although these different results may be related to biases, a large cohort study that used adequate methods to minimize biases also concluded that metformin use decreased the risk of CRC[92].

Saliently, Cardel *et al*[82] demonstrated, in a case-control study, that the risk of CRC was decreased by 17% (OR: 0.83, 95%CI: 0.74-0.92) among patients treated with metformin compared to that among patients not using metformin[82], while Liu *et al*[93] showed a 22% risk reduction for the development of CRC[93]. Importantly, the role of metformin for CRC prophylaxis was addressed in a prospective Japanese phase III trial that demonstrated that low metformin doses for 1-year reduced polyp formation and colorectal adenomas in non-diabetic patients at high risk for new polyps[94]. However, further studies are necessary to draw a definitive conclusion.

### Metformin and CRC treatment

Table 2 summarizes the clinical studies of metformin on DS cancers treatment. Specifically related to CRC, a Korean study of 595 patients with diabetes who had CRC with clinical stages I to IV showed that patients using metformin had higher overall survival (OS) and specific cancer survival compared to patients who did not use it[95]. In accordance, metformin use in 424 diabetic patients with CRC was associated with an OS of 76.9 mo *vs* 56.9 mo in patients not using metformin ($P$ = 0.048)[95]. After adjusting for possible confounding factors, the study showed that patients with DM2 treated with metformin had a 30% increase in OS when compared to patients with DM2 treated with other antidiabetic drugs[95]. Recent meta-analyses demonstrated that metformin increases the OS of patients with CRC, as well as a 10% reduction in the incidence of the disease[96,97]. The ASAMET trial, an ongoing randomized, phase II, double-blind, placebo-controlled trial aims to determine the effect of low-dose aspirin and metformin in patients with stage I-III CRC in reducing CRC mortality rates and adenoma recurrence[98]. The 160 patients with CRC were divided in four arms: aspirin, metformin, aspirin plus metformin and placebo for a duration of 1 year.

The radiotherapy-induced tumor response was improved with metformin in a Korean retrospective study that evaluated patients with localized rectal cancer. The diabetic patients receiving metformin had significantly more tumor regression grade 3-4 ($P$ = 0.029) and higher lymph node downstaging ($P$ = 0.006) as compared to patients not receiving the medication. However, the disease-free survival (DFS) and OS was not affected[99]. Consistently, a study with 482 patients examined the effect of metformin use on pathologic complete response (pCR) rates and outcomes in patients submitted to neoadjuvant chemoradiotherapy for rectal cancer. The pCR rates were higher in patients with DM2 taking metformin (35%) compared with those in nondiabetic patients (16.6%) and patients with DM2 not using metformin (7.5%). Additionally, significantly increased DFS and OS was found in patients taking metformin[100].

A phase II clinical trial addressed the combination of metformin with 5-FU in patients with refractory CRC. It demonstrated a disease control rate in 8 wk of 22%, with a median OS of 7.9 mo and progression-free survival (PFS) of 1.8 mo[101]. A trial of our group with a similar design that analyzed the combination of irinotecan with metformin found 41% disease control rate and OS of 8.2 mo[102]. Further randomized prospective studies are needed to establish metformin as a modern drug for the treatment of refractory CRC.

**Table 1 Selected clinical studies of metformin on digestive system cancers chemoprevention**

| Ref. | Study design and population | Inclusion criteria | Combined interventions /drugs | Main findings |
|---|---|---|---|---|
| | | | Comparison groups | Risk estimates and 95%CI |
| **Colorectal cancer** | | | | |
| Cardel *et al*[82], 2014 | Case-control study. Cases-controls: 2088:9060 | Cases: DM2 with CRC. Controls: DM2 without CRC | Metformin user *vs* nonuser | OR: 0.83 (0.68-1.00) |
| Lee *et al*[83], 2011 | Prospective Cohort, Taiwan. *n* = 480984 | DM2 and cancer free subjects | Metformin user *vs* nonuser | HR: 0.36 (0.13-0.98) |
| Sehdev *et al*[84], 2015 | Case control study. Cases-controls: 2682:5365 | Cases: DM2 with CRC. Controls: DM2 without CRC | Metformin user *vs* nonuser | OR: 0.85 (0.76-0.95) |
| Tseng *et al*[84]., 2012 | Retrospective Cohort. Men: 493704. Women: 502139 | Subjects covered by National Health Insurance without CRC | Metformin user *vs* nonuser | RR: 0.64 (0.49-0.84) |
| Zhang *et al*[86], 2011 | Meta-analysis. 108161 DM2 patients | Studies conducted in humans that evaluate metformin and CRC | Metformin user *vs* nonuser | RR: 0.63 (0.47-0.84) |
| Kowall *et al*[87], 2015 | Retrospective Cohort, United Kingdom. 80263 DM2 patients | Patients aged 30-89 years with DM2 diagnosis | Metformin user *vs* sulfonylurea user | HR: 1.04 (0.82-1.31) |
| Lin *et al*[88], 2015 | Prospective Cohort. 36270 DM2 patients. 145080 non DM2 | Patients older than 20 years old DM2 and Cancer- free | Metformin user *vs* nonuser | HR: 0.74 (0.53-1.03) |
| Smiechowski *et al*[89], 2013 | Case-control, United Kingdom. Cases-controls: 607:5837 | DM patients treated with non-insulin antidiabetic agents | Metformin user *vs* nonuser | RR: 0.93 (0.73-1.18) |
| Bodmer *et al*[90], 2012 | Case control, United Kingdom. Cases-controls: 920:5519 | Cases: DM2 with CRC. Controls: DM2 without CRC | Metformin user *vs* nonuser | Men: OR: 1.81 (1.25-2.62). Women: OR: 1.00 (0.63-1.58) |
| Knapen *et al*[91], 2013 | Retrospective Cohort, Denmark. 177281 DM2 with OHA | Oral antidiabetic drug users were matched 1:3 with population-based reference group | Biguanide user *vs* non-diabetic | HR: 1.19 (1.08-1.30) |
| Bradley *et al*[92], 2018 | Retrospective Cohort, Northern California. 47351 DM2 patients | DM2 and no history of cancer or metformin use | Long-term metformin use ( 5 years) *vs* nonuser | All population: HR: 0.78 (0.60-1.02). Men: HR: 0.65 (0.45-0.94) |
| Liu *et al*[175], 2017 | Meta-analysis. 20 case-control and cohort studies | Studies about metformin therapy and risk of adenoma/CRC in DM2 patients | Metformin user *vs* nonuser | Adenoma: OR: 0.75 (0.59-0.97). Carcinoma: OR: 0.781 (0.7-0.87) |
| Higurashi *et al*[94], 2016 | RCT, phase 3. *n* = 151 patients with resected adenomas or polyps | Non- diabetic adult patients who had previously had single or multiple colorectal adenomas or polyps resected by endoscopy | Metformin 250 daily or placebo (1:1) for 1 yr | Adenoma: RR 0.60 (0.39-0.92) |
| **Gastric cancer** | | | | |
| Tseng *et al*[107], 2016 | Retrospective Cohort, Taiwan. 287971 DM2 with metformin. 16217 DM2 without metformin | DM2 patients newly treated with antidiabetic drugs | Metformin user *vs* nonuser | HR: 0.45 (0.36-0.56) |
| Dulskas *et al*[108], 2020 | Retrospective Cohort study. *n* = 99992 | DM2 patients with gastric cancer | Metformin user *vs* nonuser | SIR: 0.75 (0.66-0.86) |
| Ruiter *et al*[109], 2012 | Retrospective Cohort study. 85289 DM2 patients | DM2 with more than one prescription of antidiabetic drugs | Metformin user *vs* sulfonylurea user | HR: 0.90 (0.88-0.91) |
| Kim *et al*[110], 2014 | Retrospective cohort study. 39978 DM2 patients | DM2 receiving oral antidiabetic drugs | Metformin user and non-insulin user *vs* nonuser | HR: 0.73 (0.53-1.01) |
| Cheung *et al*[112], 2019 | Prospective Cohort study. 7266 DM2 | DM2 with prescription of therapy for H. pylori. Exclusion: history of GC | Metformin user *vs* nonuser | HR: 0.49 (0.24-0.98) |
| Tsilidis *et al*[113], 2014 | Retrospective Cohort study. 51484 metformin. 18264 sulfonylureas | DM2 receiving oral antidiabetic drugs | Metformin user *vs* sulfonylurea user | HR: 0.96 (0.60-1.56) |
| de Jong *et al*[114], 2017 | Retrospective Cohort study, Netherlands. 57621 DM2 with OHA | DM2 receiving oral antidiabetic drugs | Metformin user *vs* nonuser | HR: 0.97 (0.82-1.15) |

| | | | | |
|---|---|---|---|---|
| Zheng *et al*[115], 2019 | Prospective Cohort study. 544130 DM2 patients | Diabetes Cohort: DM2 receiving antidiabetic drugs. Matched cohort: common-medication users. Exclusion: History of GC or gastrectomy | Metformin user *vs* nonuser | Non-cardia: HR: 0.93 (0.78-1.12). Cardia: HR: 1.49 (1.09-2.02) |
| Shuai *et al*[116], 2020 | Meta-analysis. 11 cohort studies | Studies conducted in humans that evaluate metformin and GC risk | Metformin user *vs* nonuser | HR: 0.79 (0.62-1.00) |
| Zhou *et al*[117], 2017 | Meta-analysis. 7 Cohort studies. *n* = 591077 | Studies conducted in humans that evaluate metformin and GC risk | Metformin user *vs* nonuser | HR: 0.76 (0.64-0.91) |
| **Pancreatic ductal adenocarcinoma** | | | | |
| Currie *et al*[123], 2009 | Retrospective cohort study. *n* = 62.809 DM2. Comparison between treatment: Metformin alone; Sulfonylurea alone; metformin plus sulfonylurea; insulin | DM2 developed > 40 years of age; United Kingdom residents | Metformin *vs* Sulfonylurea. Metformin *vs* Insulin | HR: 0.20 (0.11-0.36). HR: 0.22 (0.12-0.38) |
| Li *et al*[124], 2009 | Hospital-based case control. Cases-controls: 973:863. Comparison between treatment: Metformin; insulin secretagogues; Other antidiabetic medications; insulin | DM subjects; cases: Newly PDAC diagnosed. Controls: Nonblood relative controls; United States residents | Metformin user *vs* nonuser | OR: 0.38 (0.22-0.69) |
| Soranna *et al*[126], 2012 | Meta-analysis of 17 case-control and cohort studies. Any cancer: 17 case-control and cohort studies; 37632 cases. PDAC: 4 case-controls and retrospective cohort studies; 1192 cases | DM2 patients exposed to metformin alone or combined to sulfonylurea | Metformin user *vs* nonuser | RR: 0.38 (0.14-0.91) |
| Zhang *et al*[125], 2013 | Meta-analysis of 37 case-control and cohort studies. *n* = 1535636 | DM2 patients on treatment | Metformin user *vs* nonuser | SRR: 0.54 (0.35-0.83) |
| **Hepatocellular carcinoma** | | | | |
| Donadon *et al*[159], 2010 | Clinic-hospitalbased case control. Cases-controls: 190:359 | Cases: HCC patients. Controls: Liver cirrhosis patients and healthy controls | Metformin *vs* sulfonylurea. Metformin *vs* insulin | OR: 0.39 (0.22-0.73). OR: 0.21 (0.11-0.42) |
| Hassan *et al*[158], 2010 | Hospital-based case control. Cases-controls: 122:86 | Cases: HCC. Controls: Healthy controls | Metformin user *vs* nonuser | OR: 0.30 (0.20-0.60) |
| Ma *et al*[160], 2017 | Meta-analysis of 19 case-control and cohort studies and post hoc analysis of RCT of DM2 patients. *n* = 550.882 | DM2 exposed to metformin or biguanide | Metformin user *vs* nonuser | OR: 0.52 (0.40-0.68) |
| **Intrahepatic cholangiocarcinoma** | | | | |
| Chaiteerakij *et al*[168], 2013 | Clinic-hospital based case-control. Cases-controls: 612:594 | Cases: ICC patients. Controls: Non-cancer patients | Metformin user *vs* nonuser | OR: 0.40 (0.20-0.90) |

PDAC: Pancreatic ductal adenocarcinoma; HR: Hazard ratio; OHA: Oxidized hyaluronate; RCT: Randomized clinical trial; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; CRC: Colorectal cancer.

Interestingly, a randomized trial that included 40 patients with stage III CRC evaluated the use of metformin in preventing oxaliplatin-induced neuropathy. After the 12$^{th}$cycle of the FOLFOX-4 regimen, in the metformin group, there were fewer patients with grade 2 and 3 neuropathy as compared to the control arm (60% *vs* 95%, $P$ = 0.009). Moreover, significantly higher total scores on the Ntx-12 questionnaire and pain score were found in the metformin arm. The serum levels of neurotensin and malondialdehyde were also significantly lower in the metformin arm after 6 and 12 cycles[103].

Furthermore, there are ongoing trials evaluating the role of metformin in CRC. We highlight, in adjuvant setting, a phase 3 trial (NCT02614339) with high-risk stage II and stage III CRC that aims to evaluate the impact of metformin for 48 mo on disease free survival. In refractory CRC setting, there is an interesting phase 2 trial is recruiting patients to explore the combination of the immune checkpoint inhibitors, such as nivolumab and metformin (NCT03800602).

### *Metformin and gastric cancer*

The effect of metformin alone or in combination with cisplatin or rapamycin was studied in a tumor xenograft model[104]. It demonstrated that metformin alone decreased tumor volume. The combination of metformin with cisplatin, rapamycin or both increase the effect of each drug alone and inhibited the peritoneal dissemination of gastric cancer (GC)[104]. In accordance, Wu performed an *in vitro* study with AGS cell lines that analyzed how the association of metformin with cisplatin or adriamycin or paclitaxel enhanced the effects of each drug alone[105]. In striking contrast, Lesan

**Table 2 Selected clinical studies of metformin on digestive system cancers treatment**

| Ref. | Study design and population | Inclusion criteria | Combined interventions /drugs | Main findings |
|---|---|---|---|---|
| **Colorectal cancer** | | | | |
| Ramjeesingh *et al*[99], 2016 | Retrospective cohort. 1394 all stages CRC patients | Patients with CRC | Metformin user *vs* nonuser | HR: 0.81 (0.60-1.08) |
| Skinner *et al*[100], 2013 | Retrospective cohort. 482 locally rectal cancer patients | Locally advanced rectal adenocarcinoma treated with chemoradiation and surgery | Metformin user *vs* nonuser | pCR: OR: 16.8 (1.6-181.1). OS at 5 and 10 years (metformin *vs* non): 81% and 79% *vs* 56% and 39% ($P$ = 0.022) |
| Miranda *et al*[101], 2016 | Phase 2 Clinical trial. 50 refractory CRC patients | Refractory CRC patients | Metformin 850 mg twice a day+ 5-FU 425 mg/m$^2$ weekly | PFS: 1.8 mo. OS: 7.9 mo. Obese *vs* lean: 12.4 *vs* 5.8 mo |
| Bragagnoli *et al*[102], 2021 | Phase 2 Clinical trial, 41 refractory CRC patients | Refractory CRC patients | Metformin 2500 mg a day+ Irinotecan 125 mg/m$^2$ D1, D8, every 21 d | PFS: 2.4 mo, CI 95%, 2.0-4.5 mo. OS: 8.4 mo, CI 95%, 5.9-10.8 mo |
| El-Fatatry *et al*[103], 2018 | Clinical Trial, 40 Stage III CRC patients | Stage III CRC patients | FOLFOX 4 12 cycles + metformin 500 mg 3 times a day | Neuropathy grade 2-3 (metformin *vs* non): 60% *vs* 95% ($P$ = 0.009) |
| **Gastric cancer** | | | | |
| Lee *et al*[118], 2016 | Retrospective Cohort, single center in Korea. 1974 GC resected patients: – 132 DM2 with metformin; –192 DM2 without metformin; –1648 non-diabetic | GC patients who underwent curative gastrectomy | Metformin user *vs* nonuser | OS-HR: 0.58 (0.37-0.93). RFS-HR: 0.63 (0.41-0.98) |
| Lacroix *et al*[120], 2018 | Retrospective Cohort. 371 Patients | Stage I to III GC patients | Metformin user *vs* nonuser | OS-HR: 0.73 (0.52-1.01); cancer specific mortality-HR: 0.86 (0.56-1.33) |
| Baglia *et al*[121], 2019 | Prospective cohort study in Shangai. 543 GC patients | Breast, CRC, lung and GC patients | Metformin user *vs* nonuser | OS-HR: 1.11 (0.81-1.53). Disease-specific survival-HR: 1.03 (0.73-1.43) |
| Seo *et al*[119], 2019 | Retrospective cohort study. 2187 GC resected patients: – 103 DM2 with metformin; –139 DM2 without metformin; –1945 non-diabetic | GC patients who underwent curative gastrectomy | Metformin user *vs* nonuser | HR: 0.45 (0.30-0.66) |
| **PDAC** | | | | |
| Sadeghi *et al*[128], 2012 | Retrospective cohort. $n$ = 302 | DM2 patients. All stages. United States single center | Metformin user *vs* nonuser | HR: 0.64 (0.48-0.86) |
| Chaiteerakij *et al*[129], 2016 | Retrospective cohort. $n$ = 980 | DM2 patients. All stages. United States single center | Metformin user *vs* nonuser | HR: 0.93 (0.81-1.07) |
| Lee *et al*[133], 2016 | Retrospective cohort. $n$ = 237 | DM2 patients. All stages. Korean single center | Use of metformin ≥ 1-mo post-diagnosis *vs* nonuser | HR: 0.61 (0.46-0.81) |
| Ambe *et al*[130], 2016 | Prospective cohort study $n$ = 44 | DM2 patients. Resected PDAC, stage I-II. United States single center | Metformin user *vs* nonuser | HR: 0.54 (0.16-1.86) |
| Cerullo *et al*[131], 2016 | Retrospective cohort. $n$ = 3393 | Resected PDAC United States population based | Metformin use after surgery *vs* nonuser | HR: 0.79 (0.67-0.93) |
| Jang *et al*[132], 2017 | Prospective cohort. $n$ = 764 | DM2, OHA user. Resected Korean population based | Metformin user *vs* nonuser | HR: 0.73 (0.61-0.87) |
| Hwang *et al*[135], 2013 | Retrospective cohort. $n$ = 516 | DM2 patients. Locally advanced and metastatic. United Kingdom population based | Use of metformin peridiagnosis *vs* nonuser | HR: 1.11 (0.89-1.38) |
| Choi *et al*[134], 2016 | Retrospective cohort. $n$ = 183 | DM2 patients. Locally advanced and metastatic. Korean single center | Metformin user *vs* nonuser | HR: 0.69 (0.49-0.97) |
| Kordes *et al*[137], 2015 | RCT, $n$ = 121 | Locally advanced and metastatic. Multicentric. Netherlands | Gemcitabine-everolimus (1000 mg/m$^2$ D1, 8, 15-every 28 d-1.000 mg/d) +/- metformin (2000 mg/d) | HR: 1.05 (0.72-1.55) |
| Reni *et al*[138], 2016 | RCT. $n$ = 60 | Metastatic. Single center. Italian | PEXG (cisplatin-epirubicin-capecitabine-gemcitabine: 30 mg/m$^2$ | HR: 1.56 (0.87-2.80) |

| | | | | |
|---|---|---|---|---|
| | | | D1,14- 30 mg/m$^2$ D1,14-2500 mg/m$^2$ D1–28 – 800 mg/m$^2$ D1–14) +/- metformin 2000 mg/d | |
| Zhou *et al*[136], 2017 | Meta-analysis12 cohort studies and 2 RCT. *n* = 94778 | Studies that investigated metformin exposition. All stages PDAC | Metformin user *vs* nonuser | HR: 0.77 (0.68-0.87) |
| Li *et al*[139], 2017 | Meta-analysis. 9 cohort study and 2 RCT. *n* = 8089 | Studies that investigated metformin exposition. All stages PDAC | Metformin user *vs* nonuser | HR: 0.86 (0.76-0.97) |
| Wan *et al*[140], 2018 | Meta-analysis 15 cohort studies and 2 RCT, *n* = 36791 | Studies that investigated metformin exposition. All stages PDAC | Metformin user *vs* nonuser | HR: 0.88 (0.80-0.97). Asians only HR: 0.74 (0.58-0.94); Stage I-II HR: 0.76 (0.68-0.86); Stage III-IV HR: 1.08 (0.82-1.43) |
| Braghiroli *et al*[141], 2015 | Single-arm phase II. *n* = 20 | Locally advanced or metastatic. 2$^{nd}$ line treatment. Single center. Brazilian | Paclitaxel (80 mg/m$^2$ D1, 8, 15 every 28 d) + metfomin 1750 mg/d | DCR at 8 wk 31, 6% |
| **Pancreatic neuroendocrine tumor** | | | | |
| Pusceddu *et al*[153], 2018 | Retrospective cohort. *n* = 445 | Locally advanced or metastatic. Multicentric. Italian | No DM2 *vs* DM2. Metformin user *vs* nonuser | HR: 0.45 (0.32-0.62). HR: 0.49 (0.34-0.69) |
| **Hepatocellular carcinoma** | | | | |
| Chen *et al*[163], 2011 | Retrospective cohort. *n* = 53 | DM2. Early-stage HCC. RFA treated. Single center. Taiwanese | Metformin user *vs* nonuser | HR: 0.24 (0.07-0.90) |
| Ma *et al*[164], 2016 | Meta-analysis. 11 cohort studies. *n* = 3452 | Studies that investigated metformin exposition. HCC patients | Metformin user *vs* nonuser | HR: 0.59 (0.42-0.83) |
| **Intrahepatic cholangiocarcinoma** | | | | |
| Yang *et al*[169], 2016 | Retrospective cohort. *n* = 250 | DM2. Newly diagnosed ICC. United States single center | Metformin user *vs* nonuser | HR: 0.80 (0.60-1.20) |

PDAC: Pancreatic ductal adenocarcinoma; ORC: Origin recognition complex; HR: Hazard ratio; PCR: Polymerase chain reaction; OS: Overall survival; PFS: Progression-free survival; RFS: Regarding refeeding syndrome; OHA: Oxidized hyaluronate; RCT: Randomized clinical trial; PEXG: Pseudoexfoliative glaucoma; DCR: Dacryocystorhinostomy; HCC: Hepatocellular carcinoma; RAF: Rapidly accelerated fibrosarcoma; ICC: Intrahepatic cholangiocarcinoma; CRC: Colorectal cancer; DM2: Diabetes mellitus type 2; GC: Gastric cancer.

*et al*[106] showed *in vitro* that metformin and cisplatin in combination decreased the effects of cisplatin alone[106].

In recent years, several observational studies have shown that metformin reduces the risk of GC[107-112]. The study of Tseng *et al*[107] demonstrated that GC risk was reduced using metformin, especially when the cumulative duration was more than 2 years[107]. In addition, metformin reduced the risk of GC, while opposite results were observed with sulfonylureas[108].

On the other hand, a study conducted in United Kingdom did not show a difference in GC incidence in patients receiving metformin compared to sulfonylureas[113]. Other reports also could not find any reduction in GC risk associated with metformin us[83,114,115]. Despite that, a meta-analysis showed a 21% reduction in the risk of GC with the use of metformin, in Asians the benefit was more prominent than in Westerners[116]. Another meta-analysis of cohort studies that included 591077 patients found a significantly lower incidence of GC with metformin therapy than other types of therapy (HR: 0.763; 95%CI: 0.642-0.905)[117].

Two retrospective studies conducted by Lee *et al*[118] and Seo *et al*[119] concluded that metformin reduced GC recurrence in patients undergoing gastrectomy[118,119]. Lacroix *et al*[120] showed that metformin improved OS but not cancer specific survival, in contrast, Baglia *et al*[121] observed that metformin use did not impact patient's survival[120,121].

More studies are needed to confirm the effect of metformin in GC treatment and chemoprevention. Unfortunately, there are few clinical trials that are ongoing to analyze this question. An interesting phase 2 randomized trial (NCT04114136) are ongoing to evaluate the synergistic effect of metformin, rosiglitazone and anti-PD-1 on

the treatment of refractory solid tumors including GC. Metformin could reduce tumor oxygen consumption creating a less hypoxic T cell environment leading to restore its anti-tumor cell function. The trial NCT04033107 analyze the combination of metformin and vitamin C in DS tumors including GC.

### Metformin and pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer death in the United States and its prognosis remains dismal, encouraging research to discover innovative agents active in its treatment is an urgent unmet need[122]. Pancreatic ductal adenocarcinoma (PDAC) is its most common histologic type. An association between metformin use and decreased PDAC incidence in patients with DM2 was first recognized by two large clinical studies. In a large general practice retrospective cohort, Currie *et al*[123] reported risk reduction in metformin users related to sulfonylurea users (HR: 0.20; 95%CI: 0.11-0.36) and to insulin-based-treatment users (HR: 0.22; 95%CI: 0.12-0.38). Likewise, in a hospital-based case-control study, Li *et al*[124] encountered risk reduction in metformin users compared to those who did not use metformin (OR: 0.38; 95%CI: 0.21-0.67). Several meta-analyses have strongly reinforced PDAC risk reduction with metformin use in patients with DM2[18,125-127]. However, this effect should prospectively be confirmed in large prospective clinical trials.

Regarding survival, in a retrospective study, Sadeghi *et al*[128] reported a 36% lower risk of death (HR: 0.64; 95%CI: 0.48-0.86), OS benefit of 4 mo (15.2 mo *vs* 11.1 mo) and approximately 2-fold increase in 2-year survival rate (30.1% *vs* 15.4%) in patients who took metformin compared to those inpatients who did not take metformin. Interestingly, longer survival was only observed in non-metastatic disease, when stratified by disease stage[128]. Further evidence also encountered survival improvement in the subgroups of resected or locally advanced but not in patients with metastatic disease[129]. Specifically, among resected patients with PDAC, metformin use seemed to improve OS after 18 mo[130-132]. Related to locally advanced or metastatic disease, further evidence was contradictory on survival gains in patients with PDAC exposed to metformin with benefit being reported only in an Asian cohort[133-135]. A large meta-analysis analyzed data from 12 retrospective cohorts demonstrating OS improvement in metformin users at various stages (HR: 0.77; 95%CI: 0.68-0.87)[136].

Stimulated by this retrospective evidence, two European groups explored, in randomized clinical trials (RCTs), the association of metformin with gemcitabine-based chemotherapy as first-line treatment of advanced PDAC with negative results on OS improvement[137,138]. Recently, a meta-analysis, with inclusion of two RCTs, re-analyzed the improvement in OS and confirmed benefit in the whole population of diabetic patients with PDAC (HR: 0.86; 95%CI: 0.76-0.97)[139]. Analysis of subgroups in this study demonstrated improved survival in patients with resected or locally advanced tumors but not in the metastatic group. Similar results were observed in another group with a benefit in OS at various stages, which was more evident in the subgroups of less advanced stages and Asian patients[140]. Considering second-line treatment, a single arm prospective study did not reach survival gain of metformin associated to paclitaxel[141]. Results of ongoing clinical trials recently completed are expected with substantial interest. NCT01666730 explores overall survival improvement of metformin associated with modified FOLFOX6 in metastatic patients, NCT02005419 evaluates DFS at 1 year with the combination of metformin and gemcitabine in resected subjects and NCT02048384 analyses safety of metformin with or without rapamycin after disease stabilization on first line chemotherapy in metastatic individuals.

This clinical evidence is associated with the pre-clinical data that pancreatic cancer cells are sensitive to inhibition of oxidative phosphorylation, decreases in insulin-IGF signaling and inhibition of the mTOR pathway through AMPK activation, which are some of the major antineoplastic effects of metformin[39,142-145]. Identifying predictive or prognostic factors of response to metformin should be of relevance to select patients most likely to benefit from the effects of metformin[39]. Recent advances in molecular characterization might distinguish different biology and response to therapy in patients with morphologically similar PDAC and may be incorporated into clinical trials[146-148]. Moreover, the recently experienced challenge of standard of care in advanced pancreatic cancer treatment with polychemotherapy also brings new perspectives, as patients experience longer survival with the need to combine other active agents[149,150]. Future trials would include disease stage, identification of biomarkers and concentrations of metformin in neoplastic tissue to powerfully evaluate the benefit of metformin in the treatment of PDAC.

Another pancreatic neoplasm with rising incidence is pancreatic neuroendocrine tumors (panNETs)[151]. Few studies have evaluated the clinical benefit of metformin in the treatment of panNETs[152]. Pusceddu *et al*[153], in a multicentric retrospective cohort of patients receiving everolimus with or without somatostatin analogues, reported increased PFS in diabetic patients exposed to metformin compared to diabetic patients not exposed to metformin or non-diabetic patients [44.2 *vs* 20.8 mo (HR: 0.49; 95%CI: 0.34-0.69) or 15.1 mo (HR: 0.45; 95%CI: 0.32-0.62), respectively][153]. This result correlates with *in vitro* evidence that metformin decreases proliferation in human panNET cell lines[154,155]. A recent study demonstrated that the combination of metformin and everolimus strongly inhibited human panNET cell proliferation through mTOR suppression, compared to each agent used alone[156]. Results of the ongoing NCT02294006 prospective trial are expected to better evaluate the effects of this experimental treatment on PFS at 12 mo.

### Metformin and hepatocellular carcinoma

The incidence of hepatocellular carcinoma (HCC) has strongly increased in last two decades, as well as the prevalence of its metabolic risk factors[156,157]. Hassan *et al*[158] and Donadon *et al*[159], in hospital-based case-control studies, first observed the strong association of metformin use and reduced risk of HCC in subjects with DM2 (HR: 0.15; 95%CI: 0.04-0.50) (HR: 0.30; 95%CI: 0.20-0.60)[156,158,159]. This protective effect was validated by accumulated evidence of observational studies including more than 0.5 million subjects (OR: 0.52; 95%CI: 0.40-0.68), being more evident in case-control than in cohort studies and without significance in the *post hoc* analysis of RCTs[160-162]. These data suggest an association between metformin use and reduced HCC incidence that needs to be confirmed in prospective clinical trials.

Improvement in HCC survival was first reported by Chen *et al*[163] in an early-stage cohort of patients treated with radiofrequency ablation with longer OS in metformin users compared to non-users (HR: 0.24; 95%CI: 0.07-0.90)[163]. A meta-analysis of 11 cohort studies was in accordance with better prognosis related to metformin use in patients with HCC related to their counterparts (HR: 0.59; 95%CI: 0.42-0.83)[164].

Although the antineoplastic effects of metformin in liver cancer are not completely understood, pre-clinical evidence observed inhibition of proliferation and induction of cell cycle arrest and apoptosis in HCC cells through AMPK activation[165,166]. Future prospective trials should explore the potential benefit of metformin in prevention and treatment of HCC.

### Metformin and intrahepatic cholangiocarcinoma and gallbladder cancer

Intrahepatic cholangiocarcinoma (ICC) is the second most common hepatic cancer, and its incidence has markedly increased in the last decades[167]. Chaiteerakij *et al*[168], in a clinic-hospital-based retrospective cohort, reported 60% reduced risk of ICC in patients with DM2 who used metformin related to non-users (OR: 0.4; 95%CI: 0.2-0.9)[168]. The same group, however, did not encounter better prognosis in patients with DM2 with ICC taking metformin (HR: 0.8; 95%CI: 0.6-1.2)[169]. Although gallbladder cancer (GBC) is the most common biliary tract cancer[170], no clinical data and scarce basic evidence have explored the antineoplastic effects of metformin and its potential mechanisms of action in GBC.

Regarding comprehension of the possible mechanistic effects of metformin on ICC and GBC there are some *in vitro* and *in vivo* evidence. Overall, the studies observed the induction of apoptosis and cell cycle arrest mediated by activation of the AMPK-mTOR axis[171-173]. The association of metformin in combination with gemcitabine and cisplatin (the standard of care for advanced ICC) enhanced the antiproliferative effects of treatment in a cell model through their effects on AMPK, cyclin D1 and caspase-3[174]. Furthermore, Liu *et al*[175] first observed the decreased survival of GBC cells *via* inhibition of phosphorylated Akt (p-Akt) and Bcl-2 signaling[175]. Likewise, metformin inhibited GBC cell proliferation *via* downregulation of HIF-1α and VEGF and promoted cell cycle arrest by reduction of cyclin D1 expression in different animal experiments[176,177]. The association of metformin with cisplatin also promoted reduced expression of p-Akt and cyclin D1 downregulation, resulting in a synergistic antiprolife-rative effect in GBC cells[178].

These pre-clinical and preliminary clinical evidence highlights the need for metformin to be more deeply explored in clinical studies of ICC and GBC prevention and treatment. Considering the rationale that metformin may be active in the prevention and treatment of ICC and limited clinical data, exploratory studies should address this issue for a better understanding of its benefit in these clinical settings.

## PERSPECTIVES

DS tumors are often associated to high morbidity and mortality and their incidence has increased over recent decades[179]. Recognition of its main risk factors and conditions of worse prognosis as well as development of strategies for prevention and treatment urges. In this context, projection of a worldwide burden of cancer attributable to diabetes and excess weight for the near future is an alarming public health concern[180]. Association of several cancers, including many DS tumors to diabetes and obesity have already been recognized (IARC, WCRF). Further strategies of prevention and treatment urge to be known. The large amount of evidence presented herein supports the idea of an important effect of metformin in decreasing risk and improving prognosis of several DS tumors.

Although most clinical studies presented here are retrospective that are often limited by immortal time and selection bias, recent discoveries of pre-clinical research on antineoplastic effects of metformin establish biological plausibility for the clinical data and reinforce the interest on its effects in carcinogenesis and cancer progression. These preclinical and clinical evidence supports running of adequately powered trials to investigate clinical use of metformin on DS tumors treatment. This should consider diabetic status, predictive biomarkers, disease stage and treatment setting. Concerning chemoprevention, safety, low cost, and widespread access are key to its feasibility. Therefore, repurposing metformin for DS cancer treatment is a scientific field of remarkable interest as it focuses on a global public health problem.

Currently, clinical research is considered a job with its inherent needed professional skills[181]. Taking in consideration that the low metformin cost does not impact the expensive process of drug repurposing, the development of this potential anti-cancer drug has been hampered. Moreover, the current stage of metformin clinical development needs testing in large, randomized, genome-guided, multicenter trials. These aspects explain, at least in part, the shortage of current studies on metformin in cancer prevention and treatment despite the large number of pre-clinical and clinical evidence indicating its potential benefit. We hope that this comprehensive review integrating the potential mechanisms, pre-clinical and clinical studies of metformin as anticancer agent alert the DS cancer community for the need of studying metformin effects in more specific clinical scenarios.

## CONCLUSION

The remarkable intracellular pathway change caused by oncogenesis and the potential mechanisms of the antitumoral action of metformin have been supported. They have revealed novel target molecules and newly discovered treatment possibilities. In connection with epidemiological, pre-clinical, and clinical research, data support that metformin benefits some patients with DS tumors, requiring strict clinical trials to identify those who might obtain advantage from metformin combinations. Given that the survival outcomes are affected by a multitude of factors, such as cancer type, differentiation, staging and treatment, for adequately repurposing the use of metformin in DS cancers it is essential to take into consideration patient characteristics that may serve as predictive biomarkers of metformin antitumoral effects, such as insulin resistance, diabetes, body composition, and chronic diseases related to inflammation, as well as the specific tumor driven oncogenic pathway, which may interfere with the direct and indirect antitumoral effects of metformin.

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*REVIEW*

# Mesenchymal stromal cell secretome in liver failure: Perspectives on COVID-19 infection treatment

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## Abstract

Due to their immunomodulatory potential and release of trophic factors that promote healing, mesenchymal stromal cells (MSCs) are considered important players in tissue homeostasis and regeneration. MSCs have been widely used in clinical trials to treat multiple conditions associated with inflammation and tissue damage. Recent evidence suggests that most of the MSC therapeutic effects are derived from their secretome, including the extracellular vesicles, representing a promising approach in regenerative medicine application to treat organ failure as a result of inflammation/fibrosis. The recent outbreak of respiratory syndrome coronavirus, caused by the newly identified agent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has forced scientists worldwide to use all available instruments to fight the infection, including the inflammatory cascade caused by this pandemic disease. The use of MSCs is a valid approach to combat organ inflammation in different compartments. In addition to the lungs, which are considered the main inflammatory target for this virus, other organs are compromised by the infection. In particular, the liver is involved in the inflammatory response to SARS-CoV-2 infection, which causes organ failure, leading to death in coronavirus disease 2019 (COVID-19) patients. We herein summarize the current implications derived from the use of MSCs and their soluble derivatives in COVID-19 treatment, and emphasize the potential of MSC-based therapy in this clinical setting.

**Key Words:** Mesenchymal stromal cell; COVID-19; SARS-CoV-2; Organ failure;

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**Core Tip:** The recent coronavirus disease 2019 (COVID-19) pandemic outbreak has forced scientists worldwide to use all available options to fight this disease, in particular the inflammatory cascade caused by this infection. Mesenchymal stromal cells, for their immunomodulatory potential, represent a valid approach to combat organ inflammation. The main targets for this virus are the lungs, while other organs such as the liver are compromised by the infection. Evaluation of the albumin role in COVID-19 patients, and the connection to the "capillary leak syndrome" have focused attention on liver dysfunction correlated with the infection.

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## INTRODUCTION

The liver can be damaged by various factors, including cytotoxic molecules, ischemia, metabolic alterations, or viral infections[1], which result in inflammatory responses contributing to further liver damage[2]. If the inflammation persists, a transition from acute to chronic injury can occur, inducing hepatic fibrosis[2]. Therefore, therapies that can reduce liver inflammation/fibrosis are crucial in order to avoid organ failure and the need for transplantation.

In recent years, the use of mesenchymal stromal cells (MSCs) has been considered a promising therapeutic approach to treat liver injuries[3]. MSCs can be isolated from different compartments including adipose tissue[4], umbilical cord[5], bone marrow[6], or placenta[7,8]. These cells have been successfully used in different therapeutic applications aimed at reducing inflammatory responses[9]. Moreover, the infusion of MSCs immediately after liver transplantation promotes organ regeneration and prolonged recipient survival by reducing acute inflammation[10].

Despite their beneficial properties, there are several limitations to the use of MSCs for cellular therapies; for example, their plasticity causes the potential risk of differentiation into undesired tissues and the possibility of malignant transformation is under debate[11,12]. To overcome these issues, the use of cell-free therapy is gaining considerable attention as a treatment for liver injury, an alternative to conventional cell transplantation[13]. Indeed, the regenerative properties of the MSC secretome include immunomodulatory effects mediated by growth factors and cytokines, such as transforming growth factor beta (TGF-β), prostaglandin E2, indoleamine 2,3-dioxygenase, hepatocyte growth factor (HGF), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α)[14,15], which can also attenuate fibrogenesis. In addition, the MSC therapeutic effects could also result from the released extracellular vesicles (EVs). EVs include a highly heterogeneous group of vesicles of different size able to modulate the immune responses[16,17]. Indeed, MSC-derived EVs can be selectively enriched with anti-fibrotic[18] and anti-apoptotic[19] factors, as well as specific non-coding RNA with therapeutic potential[20].

In December 2019, several cases of death from pneumonia were reported in Wuhan, later related to a new coronavirus-related disease called coronavirus disease 2019 (COVID-19). Analysis of its genome revealed it to be phylogenetically related to severe acute respiratory syndrome coronavirus (SARS-CoV)[21], and for this reason it was named SARS-CoV-2 by the World Health Organization (WHO). Due to its worldwide spread, the WHO declared COVID-19 a pandemic in March 2020. Angiotensin-converting enzyme 2 receptor (ACE2), highly expressed in the respiratory tract, was considered the main SARS-CoV-2 viral attachment for animal cells. Most likely for this reason, the lungs are the principal target organs for SARS-CoV-2[22,23]. This virus triggers an exacerbated immune reaction because large amounts of different

inflammatory factors, including cytokines and chemokines, are produced by immune reactive cells.

It has been hypothesized that MSC-based therapy for COVID-19 patients can prevent the development of a cytokine storm by activating the immune system and promoting organ repair[24,25]. Intravenously injected MSCs reach the lungs, where they engraft and secrete a variety of soluble factors including anti-inflammatory factors, angiogenic factors, and EVs[26,27]. Studies aimed toward reversing COVID-19 side effects through MSC treatment are ongoing. In this review, we summarize the therapeutic potentials of the MSC secretome for treating liver injuries associated with COVID-19.

## MSC SECRETOME AND EVS FOR ORGAN INJURY

The use of MSC-based therapy for regenerative medicine applications counts in the hundreds of registered clinical trials (www.clinicaltrial.gov) because of the ability of these cells to promote immunomodulation and organ regeneration[28]. The release of trophic factors has demonstrated that their action is in part attributable to their secretome and, in particular, to secreted EVs[29]. Because of their intrinsic therapeutic potential, EVs are a powerful tool of regenerative medicine for the treatment of a wide range of diseases[30]. Due to heterogeneity in size and contents, as well as lack of specific markers, distinguishing the various EV subtypes is an ongoing challenge. According to the International Society for Extracellular Vesicles (ISEV), the generic term EVs includes nano-sized particles naturally released into the extracellular space by all cell types; they are delimited by a lipid bilayer and cannot replicate[31]. The ISEV consensus suggests considering physical parameters (*e.g.*, size or density) to distinguish "small" EVs, often referred as "exosomes" (< 100-200 nm in diameter) from "medium/large" EVs or "microvesicles" (> 200 nm). EVs are replete with diverse proteins, lipids, carbohydrates, and nucleic acids, and exert many of their functions of intercellular communicators by transferring their cargo molecules among cells. The specific cargo composition of EVs is largely defined by the tissue/cell type from which they originate[32]. Similarly to EVs from other cell types, MSC-EVs can be characterized according to the guidelines indicated by the ISEV. The available data suggest that EVs may significantly contribute to the paracrine effects of MSCs on tissue regeneration[33]. Because of EVs' broad biological functions, as well as their ability to transfer molecules between cells, MSC-EV-based therapy represents an attractive alternative to cell-based therapy. Application of MSC-EVs as a cell-free therapy has several advantages over conventional cell therapy. Primarily, EV injection carries lower safety risks because of their minimal reactivity to the immune system, and seem to be generally well tolerated, even when used xenogenically[34]. Then, because of their small size compared to MSCs, the intravenous delivery of EVs presents lower risk of vascular obstructions. Finally, EVs can also be genetically manipulated to carry desired therapeutic cargo for a broad, expanding range of potential clinical applications. The number of studies demonstrating the therapeutic potential of MSC-EVs in different disease models is growing rapidly. The beneficial effects of MSC-EV-based treatment are evidenced especially in cardioprotection and angiogenesis[35].

Understanding the mechanisms of action behind the therapeutic effects of MSC-EVs are crucial in view of their future clinical applications. Despite increasing interest, this field is still in its infancy in identifying the relevant bioactive molecules released by MSC-EVs that play a role in tissue repair. Efforts to identify these molecules lead to the conclusion that MSC-EVs preferentially contain mRNAs and microRNAs (miRNAs) targeting genes that participate in several cellular pathways involved in tissue repair, such as angiogenesis, migration, proliferation, self-renewal, differentiation, cellular transport, and apoptosis[36,37]. The overexpression of certain miRNAs can contribute to enhancing the therapeutic efficacy of MSC-EVs. For example, MSC-EVs over-expressing miR-21 have neuroprotective effects by targeting several genes involved in the inhibition of cell apoptosis[38,39]. The list of miRNAs known to increase the therapeutic potential of MSC-EVs in numerous disease models is long, and their therapeutic effects range from tumor modulation, immune suppression, and angiogenesis to tissue regeneration[40].

In addition to miRNAs, the beneficial effect of EV-derived proteins has been explored in terms of tissue repair and anti-inflammatory effects as a treatment for liver fibrosis, ischemia, myocardial infarction, acute renal injury, neural regeneration, or in the context of bone and cartilage regeneration[40]. Proteins identified in MSC-EVs and

linked to tissue repair include glial-derived neurotrophic factor, vascular endothelial growth factor, fibroblast growth factor, HGF, and angiotensin 1[41].

Although the number of clinical studies is limited, growing evidence shows the beneficial effects of MSC-EVs on tissue injuries. The impact of MSC-EVs on tissue regeneration has been investigated in several animal models of neuronal, cardiac, bone, cartilage, kidney, muscle, wound healing, respiratory injury, and liver regeneration[41,42]. Interestingly, data from animal models indicate that MSC-EVs can exert therapeutic potential similar to their cellular origin[41,43-46]. The list of registered clinical trials (https://clinicaltrials.gov) reporting tissue injuries-treated with MSC-EVs is shown in Table 1.

MSC-EVs show great potential as a regenerative medicine treatment for liver diseases. The benefits of MSC-EVs in liver diseases are documented in animal models of both acute[20] and chronic[47] liver injuries. MSC-EVs exert a beneficial effect by alleviating fibrosis and improving regeneration of hepatocytes[46]. In particular, EVs from fetal MSCs promote hepatocyte proliferation and decrease hepatocyte apoptosis in liver injury induced by carbon tetrachloride[48], or ameliorate oxidative stress in ischemia reperfusion injury (IRI) models in rats[49] and mice[50]. Similarly, EVs of MSC-derived induced pluripotent stem cells have hepatoprotective effects on a rat model of IRI by inducing hepatocyte proliferation[51,52]. Finally, the anti-fibrotic effects of hydrogel-embedded MSC-EVs are documented in chronic liver failure[53]. The results from *in vivo* studies indicate EVs as essential contributors to MSC therapeutic efficacy, and suggest that MSC-EV-based therapy may be a successful alternative to cell-based treatments. Nevertheless, there are still many important questions to be answered before MSC-EVs can become a fully realized cell-free therapy. These challenges comprise studies establishing the exact contribution of EVs to MSC-based therapy, including the underlying molecule mechanisms, or identifying which EV population is the most therapeutically effective. In addition, a major ongoing debate in the field of MSC EV-based therapy concerns the purity of the obtained vesicles due to contamination of the samples with non-EV proteins, RNAs, and lipoproteins[41].

## LIVER FAILURE IN COVID-19 PATIENTS

SARS-CoV-2 is the etiological agent of the pandemic COVID-19, characterized by respiratory distress and/or hypoxemia, fever, fatigue, dry cough and, in severe cases, septic shock, metabolic acidosis, and death[54]. SARS-CoV-2, as with other corona viruses, enters the host cells by binding to the ACE-2 receptor[55], while the serine protease transmembrane serine protease 2 is required for S protein priming[56]. Despite the higher tropism for the respiratory tract, SARS-CoV-2 also targets other tissues, given that the ACE2 receptor is widely distributed in other tissues[57-61]. To shed light on the SARS-CoV-2 tropism, Nardo *et al*[62] analyzed, on the Human Protein Atlas, the expression levels of two proteins, ACE-2 receptor and TMPRSS2, in different human tissues, thus revealing a higher expression in the intestine and gall bladder, but their absence in the liver. A single-cell analysis, performed on healthy human liver samples, showed that while ACE-2 expression level in cholangiocytes is comparable to that of alveolar cells in the lungs, it is barely detectable in hepatocytes[63]. Interestingly, the liver cell line HuH7 is an established permissive cell type for both SARS-CoV and SARS-CoV-2 infection, and has recently been extensively used as a model in SARS-CoV-2 studies[64,65]. In addition, an *in vitro* study found that SARS-CoV-2 infection leads to a decrease of cholangiocellular tight junction protein claudin 1 mRNA expression, implying a reduced barrier function of cholangiocytes[66]. The presence of SARS-CoV-2 receptors in the gastrointestinal (GI) tract suggests an important role of the hepatobiliary tract in viral replication and excretion[67]. In fact, the virus has also been isolated from stool samples[68]. The involvement of the GI tract in COVID-19 disease is confirmed by the GI symptoms occurring in more than 60% of infected patients, as la ack of appetite, loss of smell and taste, anorexia, diarrhea, abdominal pain, nausea, and vomiting[69-74]. Moreover, post-mortem biopsies of SARS-CoV-2-infected patients showed the presence of the viral genome in hepatocytes and the GI tract by reverse transcription polymerase chain reaction (RT-PCR)[75-77].

Though liver failure in COVID-19 patients has been considered marginal, the incidence of hepatic tissue injury in these patients ranges from 14.8% to 53%[78], while mortality ranges from 58.06% to 78%[79,80]. The liver is a key organ in nearly all metabolic processes, has immunologic functions, and is the main detoxifying organ.

**Table 1 List of registered clinical trials on the use of mesenchymal stromal cell-derived extracellular vesicles for tissue injury**

| Tissue injury disease | Condition | Treatment | Trial ID | Status |
|---|---|---|---|---|
| Chronic lung disease | Pediatric bronchopulmonary dysplasia | BM-MSC-derived EVs | NCT03857841 | Phase I |
| Lung disease | Pneumonia, COVID-19 | BM-MSC-derived EVs | NCT04493242 | Not yet recruiting |
| Lung disease | Pneumonia, COVID-19 | Inhalation of mesenchymal stem cell exosomes | NCT04276987 | Phase I |
| Multiple organ failure | Multiple organ dysfunction syndrome | MSC exosomes | NCT04356300 | Not yet recruiting |
| Lung disease | Pulmonary infection | MSC exosomes | NCT04544215 | Recruiting |
| Dry eye | GVHD | UC-MSC exosomes | NCT04213248 | Recruiting |
| Cartilage injury | Osteoarthritis | Secretome or EVs from adipose MSCs | NCT04223622 | Not yet recruiting |
| Skin disease | Dystrophic epidermolysis bullosa | BM-MSC EVs | NCT04173650 | Phase II |
| Brain | Cerebrovascular disorders | Allogenic MSCs enriched with miR-124 | NCT03384433 | Phase II |

BM: Bone marrow; COVID-19: Coronavirus disease 2019; EV: Extracellular vesicle; GVHD: Graft-*vs*-host disease; MSC: Mesenchymal stromal cell; UC: Umbilical cord.

Moreover, because of the production of albumin, acute phase reactants and coagulation factors, the liver can strongly affect the multisystem manifestations of COVID-19[62]. In fact, modified levels of hepatic function indicators such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transferase, and bilirubin have been observed in patients with COVID-19, and principally in severe diseases[59,81-84]. Many studies have shown that liver injury occurs in the early stage of the disease, with mild or moderate increase of ALT, AST, or bilirubin together with a decrease in albumin levels[79,85,86]. High AST levels have been associated with the highest mortality risk[57,87], while decreased albumin levels have been associated with severe infection and poor prognosis[88,89]. Since the specific pathogenetic mechanism by which the virus causes liver injury is still unclear, many hypotheses have been offered, including immune-mediated damage. The triggering of an exacerbated immune response to the viral infection leads to a massive release of cytokines and inflammation mediators known as cytokine storm, which is responsible for immune-mediated liver damage[89] (Figure 1).

High levels of cytokines and chemokines (*i.e.* IL-1β, Il-2, IL-6, IL-8, IL-10, Il-17, interferons [IFNs], IFN-induced protein 10, TNF-α, granulocyte-macrophage colony-stimulating factor [GM-CSF], monocyte chemoattractant protein-1, macrophage inflammatory protein-1α) and other inflammatory molecules (PCR, ferritin, lactate dehydrogenase, D-dimer) have been observed in severe SARS-CoV-2-infected patients[54,57,88,90-92]. This highly inflammatory milieu leads to multiorgan damage, including liver failure, and is strictly linked to poor prognosis and death in COVID-19 patients[88,90]. As confirmation, liver samples from COVID-19 patient autopsies have revealed micro-vesicular steatosis and inflammation[93-95]. In fact, SARS-CoV-2 infects both hepatic cells and bile duct epithelium, causing liver impairment by direct cytopathic effect, as demonstrated by high transaminase levels and post-mortem liver biopsy specimens showing moderate micro-vesicular steatosis and mild lobular and portal activity[79]. Furthermore, the presence of SARS-CoV-2 has been found in parenchymal cells and vascular endothelium of the liver in COVID-19 patients[76,77].

Additional causes of liver injury can include hypoxia, hypovolemia, and microvascular thrombosis. The hypoxic state associated with COVID-19 can induce ischemic/hypoxic liver injury[87-89]. Considering that COVID-19 patients suffer from severe hypoxia, with the induction of ACE2 receptor expression on hepatocytes[96], a direct infection of hepatocytes by SARS-CoV-2 in hypoxic conditions has been suggested[25]. Liver injury can also be drug-induced. Most of the drugs used against SARS-CoV-2 are potentially hepatotoxic: Antivirals (lopinavir/ritonavir, remdesivir, umifenovir), antibiotics (macrolides, quinolones), chloroquine, tocilizumab, and steroids as well as antipyretic drugs used for fever in COVID-19[79,90,97,98]. Moreover, it must be considered that the majority of COVID-19 patients developing liver complications have a pre-existing chronic liver disease, rendering them more susceptible to liver injury. Interestingly, it has been reported that liver fibrotic/cirrhotic conditions lead to an increase of ACE-2 receptor expression in hepatocytes[96], thus suggesting again a possible role of pre-existing pathological liver

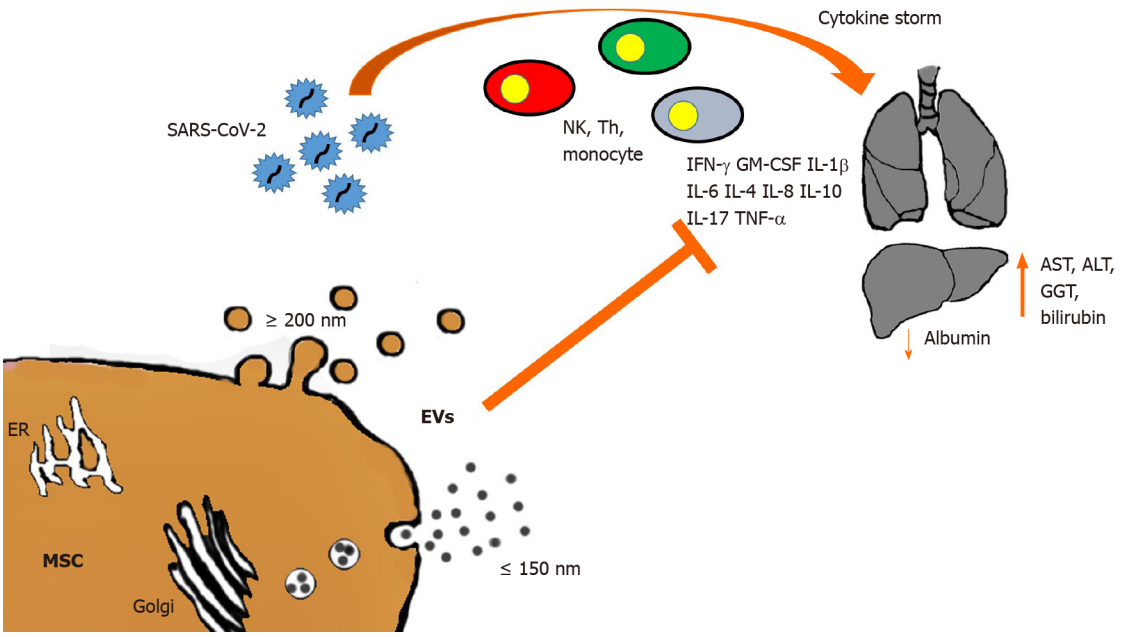


**Figure 1 Schematic representation of severe acute respiratory syndrome coronavirus 2 impact on lungs and liver.** Cytokine storm with the cascade triggered by natural killer (NK) cells, T helper (Th) cell and monocytes, and the production of inflammatory cytokines (interleukin 1 beta [IL-1b], Il-2, IL-6, IL-8, IL-10, Il-17, interferons [IFNs], IFN-induced protein 10, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor [GM-CSF]). The infection in the liver causes an increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and bilirubin, and a decrease in albumin. Mesenchymal stromal cells (MSCs) can reduce the inflammatory response by extracellular vesicle (EV) release (large ≥ 200 nm and small ≤ 150 nm). ER: Endoplasmic reticulum; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

conditions in exacerbating SARS-CoV-2 hepatic tropism.

## MSCS AND IMMUNOMODULATION IN COVID-19

The immunomodulatory properties of MSCs represent a promising therapeutic approach for the treatment of autoimmune and inflammatory diseases[99]. The anti-inflammatory and regenerative properties of MSCs have been established in numerous preclinical models of immune-related disorders including graft-*vs*-host disease, sepsis, inflammatory bowel disease, and allergic airway disease[100-103]. Recent phase I/II clinical trials have shown that the infusion of MSCs immediately after liver transplantation promoted organ regeneration and prolonged recipient survival by reducing acute inflammation, thus suggesting that MSCs can be a promising candidate for cell-based immunotherapy in solid organ transplantation[10,104]. In addition, murine models of liver fibrosis showed that human MSC-derived EVs are able to reduce hepatic inflammation and fibrosis through a decrease of TGF-β, IFN-γ, IL-1, IL-2 and TNF-α levels, an increase of Treg numbers, and a reduction of collagen deposition, all acting together to combat necrosis in the liver[105-107] (Figure 1). Among others, liver injury has been reported as a common complication in SARS-CoV-2 infection, with the degree of liver damage strictly related to the severity of COVID-19[92,108-110]. Although the exact mechanism of liver injury in COVID-19 patients is still unknown, it has been suggested that either the progression of pre-existing hepatic diseases or a direct damage of the liver can be associated with the systemic inflammation caused by SARS-CoV-2 infection, toxicity of anti-viral drugs, or hypoxia-reperfusion injury[109,110].

Pathogenic T cells are rapidly activated after SARS-CoV-2 infection, thus producing GM-CSF, IL-6, and other proinflammatory factors. GM-CSF will further activate inflammatory monocytes ($CD14^+CD16^+$), which in turn produce a larger amount of IL-6 and other pro-inflammatory factors, triggering the cytokine storm, which is the main cause of the organ damage, such as in the lungs, kidney, and liver[110]. Recently, the use of MSCs has been proposed as a promising therapeutic approach for COVID-19 patients. The effectiveness and safety of MSC-based treatment are supported by several clinical studies, suggesting that MSC therapy may improve the clinical outcomes of COVID-19 patients through immunomodulation, regulation of

inflammatory response, and promotion of tissue repair[111-115]. Moreover, a vast number of clinical trials that use MSCs to treat COVID-19 have already been registered (http://www.chictr.org.cn; https://clinicaltrials.gov). According to their immunomodulatory properties, the use of MSC-based therapies could be a novel strategy to counteract the harmful effects on the liver caused by SARS-CoV-2 infection.

## DISCUSSION

Among the numerous drug treatments, which include antiviral therapy, cytokine inhibitors (*e.g.*, IL-6), and specific antibody treatment (serum/monoclonal)[116], MSCs represent a potential option for critical cases[117]. As discussed above, SARS-CoV-2 infection induces a cytokine storm, causing acute respiratory distress syndrome and multiple-organ failure. IL-6 inhibition by tocilizumab was positively tested in a randomized clinical trial (http://www.chictr.org.cn/showprojen.aspx?proj=49409). Likewise, in this inhibition MSCs can represent a valid alternative, and it has been shown that EV administration counteracts IL-6-induced acute liver injury (ALI) in rat models through the presence of miR-455-3p[118]. MSC treatment showed that the symptomatology of patients was relieved within 2-4 d after MSC infusion, with oxygen saturation increasing to 95% at rest[119]. Another study involved critically ill COVID-19 patients treated with an infusion of human umbilical cord MSCs. In this case, the patients were treated with three different infusions of cells at an interval of 3 d, displayed no observable side effects, and were able to walk within 4 d[115]. Leng *et al*[119] showed that after infusion of MSCs in COVID-19 patients, the number of peripheral lymphocytes increased, while the levels of C-reactive protein decreased. In addition, in MSC-treated COVID-19 patients compared with those treated with conventional therapy a clear reduction of the major pro-inflammatory cytokine TNF-α, and an increase of IL-10 concentration were observed[119]. Therefore, in an immune-mediated disease condition like COVID-19 infection, the anti-inflammatory activities of MSCs could contribute to improving the conditions of patients after their infusion.

Despite the limited published data, and based on various studies, it could be speculated that SARS-COV-2 induces ALI[79]. SARS-CoV-2 could insult the liver either directly, by the cytopathic effect of the virus after infections of the hepatocytes, or indirectly, by induction of uncontrolled immune reaction, oxidative stress, and/or by pharmacological treatments for COVID-19 that induce liver injury. However, the mechanisms underlying liver impairment in COVID-19 patients are still unknown. Tian *et al*[94] found sinusoidal dilatation and focal macrovesicular steatosis in liver biopsies obtained post-mortem from four patients with COVID-19 and, in one of these, SARS-CoV-2 RNA was isolated from liver tissue. Wang *et al*[73] found that four patients (2.9% of 138 patients hospitalized for COVID-19) had chronic liver disease. In another study, cases of ALI were reported in 13 of 274 patients (4.7%)[120]. Interestingly, Richardson *et al*[121] showed that, in a study including 5700 COVID-19 patients, 58.4% and 39% developed higher levels of ALT and AST, respectively. In addition, among these patients, 56 (1%) developed acute hepatic injury (32320003). Therefore, many COVID-19 patients showed higher levels of both ALT and AST, and mainly in patients with severe disease, liver impairment can occur[54,59,120].

The intravenous administration of MSCs lowered the elevated serum levels of AST and ALT, and increased the amount of HGF, resulting in reduction of ALI[122]. Moreover, in a rat model of ALI, MSCs inhibited neutrophil infiltration, oxidative stress, and hepatocyte apoptosis[123], showing that MSC treatment had significant systemic anti-inflammatory effects and reduction of hepatic inflammation. Moreover, MSCs can prevent lung damage not only directly, with anti-inflammatory activity, but also indirectly by supporting liver function in maintaining the plasma level of albumin (Figure 1). Johnson *et al*[124] recently underscored the interplay between albumin and SARS-CoV-2, while the importance of albumin in COVID-19 patients has also been strongly stressed by several research teams, who describe a "capillary leak syndrome" in infected patients. This extravascular leakage of intravascular fluids is induced by hypoglobulinemia[125]. A histological analysis of COVID-19 lungs in SARS-CoV-2-infected patients confirmed the presence of pulmonary vascular permeability where the endothelial cells appear swollen[126]. Hypoalbuminemia is an indication of liver dysfunction in the elderly, where it is, per se, an index of increased mortality[127]. The large amounts of extravascular fluid due to the resulting vascular permeability, require mechanical ventilation to overcome the problem.

## CONCLUSION

At present, there is no standardized therapy for COVID-19 patients. Though many innovative treatments have been rapidly approved, additional experimental therapies are necessary to treat the worse cases of infection. Despite the fact that all MSC clinical trials for COVID-19 treatment are currently focused on lung/respiratory function, and some of the exclusion criteria are liver disease/insufficiency, we believe, on the basis of current studies, that MSC-based therapy can also help liver dysfunction correlated with SARS-CoV-2 infection.

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*MINIREVIEWS*

# Artificial intelligence applications in inflammatory bowel disease: Emerging technologies and future directions

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## Abstract

Inflammatory bowel disease (IBD) is a complex and multifaceted disorder of the gastrointestinal tract that is increasing in incidence worldwide and associated with significant morbidity. The rapid accumulation of large datasets from electronic health records, high-definition multi-omics (including genomics, proteomics, transcriptomics, and metagenomics), and imaging modalities (endoscopy and endomicroscopy) have provided powerful tools to unravel novel mechanistic insights and help address unmet clinical needs in IBD. Although the application of artificial intelligence (AI) methods has facilitated the analysis, integration, and interpretation of large datasets in IBD, significant heterogeneity in AI methods, datasets, and clinical outcomes and the need for unbiased prospective validations studies are current barriers to incorporation of AI into clinical practice. The purpose of this review is to summarize the most recent advances in the application of AI and machine learning technologies in the diagnosis and risk prediction, assessment of disease severity, and prediction of clinical outcomes in patients with IBD.

**Key Words:** Artificial intelligence; Machine learning; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Clinical outcomes



**Core Tip:** The application of artificial intelligence (AI) in the field of inflammatory bowel disease (IBD) has grown significantly in the past decade. AI has been used to analyze genomic datasets, construct IBD risk prediction models, and increase IBD diagnosis precision. Machine learning has been used to analyze endoscopic images to

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improve disease severity grading. AI has enabled the integration of large clinical and laboratory datasets with gene expression profiles to predict clinical outcomes such as therapy response. Future studies will need to validate these findings in independent cohorts and determine whether applying these AI-derived prediction models improves clinical outcomes in IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder of the gastrointestinal tract. IBD has emerged as a global disease with increasing incidence worldwide and associated with significant healthcare utilization[1,2]. The pathogenesis of IBD is complex and is thought to involve an interplay between loss of tolerance to commensal gut bacteria, intestinal epithelial barrier dysfunction, and immune dysregula-tion[3-7]. The diagnosis of IBD is based on a combination of factors including clinical data (*e.g.*, chronicity of gastrointestinal symptoms), laboratory values (elevated inflammatory markers such as C-reactive protein and fecal calprotectin), imaging, endoscopy, and histology (gastrointestinal inflammation with architectural distortion)[8]. Although treatment algorithms based on clinical trials and experience have been developed to inform clinical management in IBD[9], there is significant heterogeneity among patients with IBD with regards to presentation, response to therapy, and long-term clinical outcomes such development of strictures and need for surgery[10,11]. There is a great need for precision medicine strategies to improve diagno-stic and therapeutic approaches in IBD.

Precision medicine efforts in IBD have led to more in-depth phenotyping of patients with IBD using large scale databases from clinical trials and cohort studies, deep immunophenotyping using whole genome gene expression datasets, proteomics, transcriptomics, and metagenomics of gut microbiota, and complex predictive models incorporating computer-assisted analysis of endoscopic images and histology[12-14]. This has inevitably led to vast arrays of high dimensional data that pose significant challenges with traditional statistical and computational methods[15]. Technological advances in artificial intelligence (AI) have revolutionized the ability of clinicians and researchers to process, analyze, and interpret high dimensional data and large datasets.

AI is a broad and multidisciplinary field incorporating concepts from computer science, engineering, philosophy, and linguistics aimed at understanding and designing systems that display or mimic human intelligence. The term was first coined in 1965 by McCarthy J[16,17]. Machine learning (ML) is a subdiscipline of AI where computer algorithms apply statistical models to learn associations of predictive power from examples in provided datasets (*e.g.*, Dragon dictation, SPAM, Netflix). ML may be programmed through supervised learning or unsupervised learning. In supervising learning, computer programs are trained to learn associations between inputs and outputs in data through analysis of predefined outputs of interest (by human operator). Once associations have been learned using existing data, supervised ML classifiers could then be used to predict future examples using different datasets. Examples of supervised ML include random forest (RF) and support vector machines (SVM). In unsupervised learning, computer programs learn associations in data without external definitions of associations of interest. This method allows for the identification of previously undiscovered predictors. Deep learning, commonly known as neural networks, includes newer techniques that are based on models with fewer assumptions, rely on multiple layers of representation of the data with successive transformations that amplify aspects of the input which improves discrimination power and thus able to handle more complex data (*e.g.*, Facebook face recognition, credit card fraud)[17]. There has been increased interest in use of AI in IBD in recent

years with many prior groups applying ML methods to identify meaningful insights in diagnostics and prediction models in IBD. The purpose of this review is to provide a comprehensive summary of advances in the application of AI and ML technologies in the diagnosis and risk prediction, assessment of disease severity, and prediction of clinical outcomes in patients with IBD.

## LITERATURE SEARCH

We performed a literature review using PubMed (MEDLINE) from inception to December 15, 2020 of studies applying AI in IBD. Our search strategy included the following combinations: ((((((((inflammatory bowel disease[Title])) OR (ulcerative colitis[Title])) OR (Crohn's disease[Title])) AND (artificial intelligence[Title])) OR (computer-assisted[Title])) OR (computer-aided[Title])) OR (neural network[Title])) OR (machine learning[Title])) OR (deep learning[Title]). We included studies that used AI in the (1) diagnosis or risk prediction of IBD, (2) assessment of disease severity in IBD, and (3) prediction of therapy response and clinical outcomes in IBD. We excluded reviews, studies with non-human subjects (animal models), or studies that did not provide objective measures of the efficacy of AI applications (*e.g.*, measures of precision, accuracy, area under the curve (AUC), sensitivity, specificity, *etc.*).

## RESULTS

Our search strategy yielded 98 studies evaluating AI in IBD of which 58 studies[18-74] met inclusion criteria and were included in the final review. About 86.2% (50/58) of studies were published within the past 5 years (2015 and later). There were 23 studies[18-39] that focused on IBD diagnosis and risk prediction, 19 studies[40-58] which evaluated disease activity, and 17 studies[45,59-74] which predicted IBD clinical outcomes (response to therapy, colonic neoplasia, post-surgical complications, quality of life, IBD well-being and emotional content). There were 22 studies with combined IBD cohorts (CD and UC), 16 studies with UC patients only, 18 studies with CD only, and 5 pediatric IBD cohorts. The most common AI classifications used were neural networks (convolutional and deep) at 32.7% (19/58 studies), RF at 29.3% (17/58 studies), and SVM at 29.3% (17/58 studies).

### *AI in diagnosis and risk prediction of IBD*

Table 1 summarizes studies included which applied AI in the diagnosis and risk prediction of IBD. There were 17 studies focused on IBD diagnosis, whereas 5 studies focused on predicting risk of IBD. Data modalities included genetic/genomic datasets ($n$ = 16 studies), imaging and endoscopic datasets ($n$ = 4), and protein expression/proteomics ($n$ = 2 studies). Some groups have used ML to develop IBD risk prediction models based on gene expression datasets. In a cross-sectional study of 180 CD patients, 149 UC patients and 90 healthy controls by Isakov *et al*[21], RF and SVM used microarray and RNA-seq data sets to classify a list of 16390 genes. Their combined IBD risk prediction model demonstrated an AUC, sensitivity, specificity, and accuracy values of 0.829, 0.577, 0.880, and 0.808, respectively. In another cross-sectional study of 18227 CD patients and 34050 healthy controls, Romagnoni *et al*[20] used gradient boosted trees and artificial neural networks to analyze gene expression profiles. Using single nucleotide polymorphisms, their final predictive model for CD achieved AUC of 0.80. Likewise, a cross-sectional study of 20 UC patients and 20 healthy controls by Duttagupta *et al*[33] used SVM to analyze microRNA profiles. Their SVM classifier measurements revealed a predictive score accuracy of 92.8%, specificity of 96.2%, and sensitivity of 89.5% in distinguishing UC patients from normal individuals.

A major challenge in IBD diagnosis is the distinction between CD and UC which is based on clinical features such as the distribution of inflammation along the gastrointestinal tract. The misdiagnosis of IBD subtype is not uncommon[74]. Distinguishing between CD and UC is clinically important as IBD subtype informs clinical management. AI has been employed to analyze molecular data to distinguish between CD and UC. In a cross-sectional study of 59 CD patients, 26 UC patients, and 42 healthy controls applying deep belief networks (DBNs) and SVM to gene expression datasets, Smolander *et al*[25] explored the diagnosis UC from CD. Using DBN only, the accuracy for diagnosis of UC was 97.06% and CD was 97.07%. Using both DBN and SVM, accuracy for diagnosis of UC was 97.06% and CD was 97.03%. In

**Table 1 Artificial intelligence in diagnosis and risk prediction of inflammatory bowel disease**

| Ref. | AI classifier *vs* comparator | IBD type | Study design and sample size | Modality | Outcome | Study results/validation cohort |
|---|---|---|---|---|---|---|
| Mossotto *et al* [18], 2017 | Support vector machines (SVM) *vs* linear discriminant | Peds CD/UC | Prospective cohort, 287 IBD patients | Endoscopic and histologic inflammation | Diagnosis of IBD | Diagnostic accuracy of 82.7% with an AUC of 0.87 in diagnosing Crohn's disease or ulcerative colitis. Validation cohort included |
| Wei *et al*[19], 2013 | SVM with gradient boosted trees (GBT) *vs* simple log odds method | CD/UC | Cross-sectional, 30000 IBD patients, 22000 healthy controls | Genetics, ImmunoChip | Risk of IBD | The SVM demonstrated very comparable performance (AUC 0.862 and 0.826 for CD and UC, respectively), whereas GBT showed inferior performance (AUC 0.802 and0.782 for CD and UC, respectively. Validation cohort included |
| Romagnoni *et al*[20], 2019 | Artificial neural networks (ANNs) *vs* penalized logistic regression (LR), and GBT | CD | Cross-sectional, 18227 CD patients, 34050 healthy controls | Genetics, ImmunoChip | Risk of IBD | Using single nucleotide polymorphisms (SNPs), final predictive model achieved AUC of 0.80. Validation cohort included |
| Isakov *et al*[21], 2017 | Random forest (RF), SVM with svmPoly), extreme gradient boosting *vs* elastic net regularized generalized linear model (glmnet) | CD/UC | Cross-sectional, 180 CD patients, 149 UC patients, 90 healthy controls | Expression data (microarray and RNA-seq) | Risk of IBD | The method was used to classify a list of 16390 genes. Each gene received a score that was used to prioritize it according to its predicted association to IBD. The combined model demonstrated AUC, sensitivity, specificity, and accuracy values of 0.829, 0.577, 0.88, and 0.808, respectively. Validation cohort included |
| Yuan *et al*[22], 2017 | Sequential minimal optimization *vs* DisGeNET (Version 4.0) | CD/UC | Cross-sectional, 59 CD patients, 26 UC patients, 42 healthy controls | Gene Expression datasets | Risk of IBD | By analyzing the gene expression profiles using minimum redundancy maximum relevance and incremental feature selection, 21 genes were obtained that could effectively distinguish samples from IBD and the non-IBD samples. Highest total prediction accuracy was 97.64% using the 1170$^{th}$ feature set. Validation cohort included |
| Hübenthal *et al*[23], 2015 | SVM *vs* RF | CD/UC | Cross-sectional, 40 CD patients, 36 UC patients, 38 healthy controls | MicroRNAs | Diagnosis of IBD | Measured by the AUC the corresponding median holdout-validated accuracy was estimated as ranging from 0.75 to 1.00 and 0.89 to 0.98, respectively. In combination, the corresponding models provide tools for the distinction of CD and UC as well as CD, UC and healthy control with expected classification error rates of 3.1 and 3.3%, respectively. Validation cohort included |
| Tong *et al*[24], 2020 | RF *vs* convolutional neural network (CNN) | CD/UC | Retrospective Cohort, 875 CD patients, 5128 UC patients | Colonoscopy Endoscopic Images | Diagnosis of IBD | RF sensitivities/specificities of UC/CD were 0.89/0.84, 0.83/0.82, and 0.72/0.77, respectively, while the values for the CNN of CD was 0.90/0.77. The precisions/recalls of UC-CD when employing RF were 0.97/0.97, 0.65/0.53, respectively, and when employing the CNN were 0.99/0.97 and 0.87/0.83, respectively. Validation cohort included |
| Smolander *et al*[25], 2019 | Deep belief networks (DBNs) *vs* SVM | CD/UC | Cross-sectional, 59 CD patients, 26 UC patients, 42 healthy controls | Gene Expression datasets | Diagnosis of IBD | Using DBN only, accuracy for diagnosis of UC was 97.06% and CD was 97.07%. Using both DBN and SVM, accuracy for diagnosis of UC was 97.06% and CD was 97.03%. Validation cohort included |
| Abbas *et al*[26], 2019 | RF *vs* network-based biomarker discovery | Peds CD/UC | Cross-sectional, 657 IBD patients, 316 healthy controls | Large dataset of new-onset pediatric IBD metagenomics biopsy samples | Diagnosis of IBD | For the diagnosis of IBD, highest AUC attained by top Random Forest classifiers was 0.77. No validation cohort included |
| Khorasani *et al*[27], 2020 | SVM *vs* recently developed feature selection algorithm (robustness-performance tradeoff, RPT) | UC | Cross-sectional, 146 UC patients, 60 healthy controls | Gene Expression dataset | Diagnosis of IBD | Our model perfectly detected all active cases and had an average precision of 0.62 in the inactive cases. Validation cohort included |
| Rubin *et al*[28], 2019 | CITRUS supervised machine learning algorithm. No comparator | CD/UC | Cross-sectional, 68 IBD patients | Peripheral blood mononuclear cells and intestinal biopsies mass cytometry | Diagnosis of IBD | An 8-parameter immune signature distinguished Crohn's disease from ulcerative colitis with an AUC = 0.845 (95%CI: 0.742-0.948). No validation cohort included |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pal *et al*[29], 2017 | Naïve Bayes and with a consensus machine learning method *vs* Critical Assessment of Genome Interpretation (CAGI) 4 method | CD | Cross-sectional, 64 CD patients, 47 healthy controls | Genotypes from Exome Sequencing Data | Risk of IBD | The AUC for predicting risk of Crohn's disease using the SNP model was 0.72. No validation cohort included |
| Aoki *et al*[30], 2019 | Deep CNN. No comparator | CD | Retrospective Cohort, 115 IBD patients | Wireless capsule endoscopy images | Diagnosis of IBD | The AUC for the detection of erosions and ulcerations was 0.958 (95%CI: 0.947-0.968). The sensitivity, specificity, and accuracy of the CNN were 88.2% (95%CI: 84.8-91.0), 90.9% (95%CI: 90.3-91.4), and 90.8% (95%CI: 90.2-91.3), respectively. Validation cohort included |
| Bielecki *et al*[31], 2012 | SVM *vs* human reader (pathologist) | CD/UC | Cross-sectional, 14 CD patients, 13 UC patients, 11 healthy controls | Raman spectroscopic imaging of epithelium cells | Diagnosis of IBD | Raman maps of human colon tissue sections were analyzed by utilizing innovative chemometric approaches. Using SVM, it was possible to separate between healthy control patients, patients with Crohn's Disease, and patients with ulcerative colitis with an accuracy of 98.90%. No validation cohort included |
| Cui *et al*[32], 2013 | Recursive SVM *vs* unsupervised learning strategy | CD/UC | Cross-sectional, 124 IBD patients, 99 healthy controls | 16S rRNA gene analysis | Diagnosis of IBD | Selection level of 200 features results in the best leave-one-out cross-validation result (accuracy = 88%, sensitivity = 92%, specificity = 84%). Validation cohort included |
| Duttagupta *et al*[33], 2012 | SVM. No comparator | UC | Cross-sectional, 20 UC patients, 20 healthy controls | MicroRNAs | Diagnosis of IBD | SVM classifier measurements revealed a predictive score of 92.8% accuracy, 96.2% specificity and 89.5% sensitivity in distinguishing ulcerative colitis patients from normal individuals. Validation cohort included |
| Daneshjou *et al*[34], 2017 | Naïve bayes, neural networks, random forests *vs* CAGI methods | CD | Cross-sectional, 64 ICD patients, 47 healthy controls | Exome Sequencing | Diagnosis of IBD | In CAGI4, 111 exomes were derived from a mix of 64 Crohn's disease patients. Top performing methods had an AUC of 0.87. Validation cohort included |
| Geurts *et al* [35], 2005 | RF *vs* SVM | CD/UC | Prospective cohort, 30 CD patients, 30 CD patients | Proteomic Mass Spectrometry | Diagnosis of IBD | Random forest model to diagnosis IBD had a sensitivity of 81.67%, specificity of 81.17%. Support vector machine model to diagnosis IBD had a sensitivity of 87.92%, specificity of 87.87%. Validation cohort included |
| Li *et al*[36], 2020 | RF *vs* ANN | UC | Cross-sectional, 193 UC patients, 21 healthy controls | Gene Expression Profiles | Diagnosis of IBD | The random forest algorithm was introduced to determine 1 downregulated and 29 upregulated differentially expressed genes contributing highest to ulcerative colitis occurrence. ANN was developed to calculate differentially expressed genes weights to ulcerative colitis. Prediction results agreed with that of an independent data set (AUC = 0.9506/PR-AUC = 0.9747). Validation cohort included |
| Wingfield *et al*[37], 2019 | RF *vs* SVM | CD | Cross-sectional, 668 CD patients | Metagenomic Data | Diagnosis of IBD | Highest RPT measure for Crohn's disease was random forest 0.60 and SVM 0.58. For ulcerative colitis, RPT was random forest 0.70 and SVM 0.48. Validation cohort included |
| Han *et al*[38], 2018 | RF *vs* LR, CORG | CD/UC | Cross-sectional, 24 CD patients, 59 UC patients, 76 healthy controls | Gene Expression Profiles | Diagnosis of IBD | The gene-based feature sets had median AUC on the validation sets ranging from 0.6 to 0.76). Validation cohort included |
| Wang *et al*[39], 2019 | AVADx (Analysis of Variation for Association with Disease) *vs* two GWAS-based CD evaluation methods | CD | Cross-sectional, 64 CD patients, 47 healthy controls | Whole Exome or Genome Sequencing Data | Diagnosis of IBD | AVADx highlighted known CD genes including NOD2and new potential CD genes. AVADx identified 16% (at strict cutoff) of CD patients at 99% precision and 58% of the patients (at default cutoff) with 82% precision in over 3000 individuals from separately sequenced panels. Validation cohort included |

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve.

a cross-sectional study of 68 IBD patients using a CITRUS supervised ML algorithm to analyze single cell immunophenotyping of peripheral blood mononuclear cells by mass cytometry, Rubin *et al*[28] demonstrated that an 8-parameter immune signature distinguished CD from UC with an AUC = 0.845 [95% confidence interval (CI): 0.742-0.948]. ML algorithms have also been applied to analyze large arrays of endoscopic images to differentiate between UC and CD. In a recent retrospective cohort study of 875 CD patients and 5128 UC patients by Tong *et al*[24] using RF and convolutional neural networks (CNNs) on endoscopic images, the precision of diagnosing UC/CD with RF and CNNs were 0.97/0.65 and 0.99/0.87, respectively. Taken together, these studies suggest that AI classifiers have high performance in diagnosing or predicting risk of IBD but have some variability with type of AI classifier and modality of data (molecular *vs* endoscopic).

### AI in assessment of disease severity in IBD

The assessment of disease activity and grading of severity in IBD could be accomplished using validated clinical symptom scores (*e.g.*, Harvey Bradshaw Index for CD, Mayo Score for UC)[75,76], biomarkers of inflammation (*e.g.*, C-reactive protein, fecal calprotectin)[77,78], endoscopic inflammation indices (*e.g.*, Mayo endoscopic score, simple endoscopic score)[79,80], and histologic scoring systems (*e.g.*, Geboes Score, Robarts Histopathology Index)[81,82]. However, these systems may be subject to recall bias, heterogeneity in patient clinical presentation, and intraobserver and interobserver variability[83]. AI has been applied to these existing systems to improve precision and accuracy of quantifying disease severity in IBD.

Table 2 summarizes studies included which applied AI in the assessment of disease severity in IBD. There were 2 studies that assessed clinical disease activity, 2 studies that assessed disease activity by biomarker (C-reactive protein), 13 studies that focused on endoscopic inflammation, and 3 studies that focused on histologic inflammation. Data modalities included electronic health records ($n$ = 2), molecular datasets ($n$ = 3), endoscopic datasets ($n$ = 11 studies), and histologic datasets *via* endomicroscopy/endocytoscopy ($n$ = 2). Using RF to integrate and analyze clinical and laboratory data from publicly available clinical trials (UNITI-1, UNITI-2, and IM-UNITI) data consisting of 401 CD patients, Waljee *et al*[42] constructed a CD remission prediction model using the week 6 albumin to C-reactive protein ratio with an AUC of 0.76 (95%CI: 0.71-0.82). Reddy *et al*[44] applied gradient boosting machines to electronic health records and predicted inflammation severity in a retrospective cohort of 3335 CD patients with a very high accuracy (AUC) = 92.82%. In a CNN analysis of colonoscopy images from a retrospective cohort of 841 UC patients by Ozawa *et al*[55], the CNN-based computer aided diagnostic system showed a high level of performance with AUC of 0.86 and 0.98 to identify Mayo 0 and 0-1, respectively. The performance of the CNN was better for the rectum than for the right side and left side of the colon when identifying Mayo 0 (AUC = 0.92, 0.83, and 0.83, respectively). Likewise, in an ordinal CNN analysis of wireless capsule endoscopy images in a retrospective cohort of 49 CD patients by Barash *et al*[50], the classification accuracy of the algorithm was 0.91 for grade 1 *vs* grade 3 ulcers, 0.78 for grade 2 *vs* grade 3, and 0.624 for grade 1 *vs* grade 2. The role of AI in grading severity of histologic inflammation in IBD has also been explored. For example, in a retrospective cohort study of 187 UC patients by Maeda *et al*[46], application of SVM to data derived from endocytoscopy to assess histologic inflammation provided diagnostic sensitivity, specificity, and accuracy of 74% (95%CI: 65-81), 97% (95%CI: 95-99), and 91% (95%CI: 83-95), respectively. These examples highlight the clinical utility, versatility, and performance of AI classifiers in grading the disease activity of IBD patients at the clinical, endoscopic, and histologic level. AI performance may be affected by location of inflammation and may be limited by ability to discriminate between subtle differences.

### AI in prediction of therapy response and clinical outcomes in IBD

The armamentarium of therapies in IBD have expanded significantly in recent years with diverse mechanisms of action ranging from biologics that inhibit proinflammatory cytokines (anti-tumor necrosis factor-α, anti-interleukin-12/23) and leukocyte trafficking to the gut (anti-α4β7) to small molecule inhibitors of the JAK-STAT signaling pathway[84-86]. Despite several IBD treatment options available to clinicians, there are no effective biomarkers or tools to predict response to therapy or to guide selection of alternative therapies after a failed response. Likewise, there is also an unmet clinical need to predict long term clinical outcomes in IBD such as colon cancer. To address these challenges, several groups have applied AI and ML algorithms to existing clinical and molecular datasets.

**Table 2 Artificial Intelligence in assessment of disease severity in inflammatory bowel disease**

| Ref. | AI classifier *vs* comparator | IBD type | Study design and sample size | Modality | Outcomes | Study results/validation cohort |
|---|---|---|---|---|---|---|
| Kumar *et al*[40], 2012 | Support vector machines (SVM) *vs* human observers | CD | Cross-sectional, 50000 images (number of patients not given) | Small bowel capsule endoscopy | Endoscopic Inflammation | Database of 47 studies including 50000 capsule endoscopy images evaluating severity of small bowel lesions. Method had good precision (> 90% for lesion detection) and recall (> 90%) for lesions of varying severity. Validation cohort included |
| Biasci *et al*[41], 2019 | Logistic regression with an adaptive Elastic-Net penalty. No comparator | CD/UC | Prospective cohort, 118 IBD patients | Transcriptomics from purified CD8 T cells and/or whole blood | Disease severity, medication escalation | A 17-gene qPCR-based classifier stratified patients into two distinct subgroups. IBDhi patients experienced significantly more aggressive disease than IBDlo patients (analogous to IBD2), with earlier need for treatment escalation [HR 2.65 (CD), 3.12 (UC)] and more escalations over time [for multiple escalations within 18 months: sensitivity=72.7% (CD), 100% (UC); negative predictive value = 90.9% (CD), 100% (UC)]. Validation cohort included |
| Waljee *et al*[42], 2019 | RF. No comparator | CD | Post-hoc analysis of prospective clinical trials, 401 CD patients | Clinical and laboratory data from publicly available clinical trials (UNITI-1, UNITI-2, and IM-UNITI) | Crohn's disease remission, C-reactive protein < 5 mg/L | A prediction model using the week-6 albumin to C-reactive protein ratio had an AUC of 0.76 [95% confidence interval (CI): 0.71-0.82]. Validation cohort included |
| Mahapatra *et al*[43], 2016 | RF. No comparator | CD | Cross-sectional, 35 CD patients | Abdominal magnetic resonance imaging | Segmentation of diseased colon (intestinal inflammation) | Model segmentation accuracy ranged from 82.7% to 92.2%. Validation cohort included |
| Reddy *et al*[44], 2019 | Gradient boosting machines *vs* logistic regression | CD | Retrospective, 3335 CD patients | Electronic medical record | Severity of intestinal inflammation (by C-reactive protein) | Machine-learning-based analytic methods such as gradient boosting machines can predict the inflammation severity with a very high accuracy (AUC) = 92.82%. Validation cohort included |
| Douglas *et al*[45], 2018 | RF. No comparator | Peds CD | Cross-sectional, 20 CD patients, 20 healthy controls | Shotgun metagenomics (MGS), 16S rRNA gene sequencing | Disease State (Relapse/Remission) | MGS modules significantly classified samples by disease state (accuracy = 68.4%, $P$ = 0.043 and accuracy = 65.8%, $P$ = 0.03, respectively), 16S datasets had a maximum accuracy of 68.4% and $P$ = 0.016 based on strain level for disease state. Validation cohort included |
| Maeda *et al*[46], 2019 | SVM *vs* human reader | UC | Retrospective cohort, 187 UC patients | Endocytoscopy | Histologic inflammation | Computer aided diagnosis (CAD) of histologic inflammation provided diagnostic sensitivity, specificity, and accuracy as follows: 74% (95%CI: 65-81), 97% (95%CI: 95-99), and 91% (95%CI: 83-95), respectively. Its reproducibility was perfect ($k$ = 1). Validation cohort included |
| Charisis *et al*[47], 2016 | SVM *vs* human reader | CD | Retrospective cohort, 13 CD patients | Wireless capsule endoscopy (WCE) images | Endoscopic Inflammation | Experimental results, along with comparison with other related efforts, have shown that the hybrid adaptive filtering [HAF-Differential Lacunarity (DLac) analysis (HAF-DLac)] *via* SVM approach evidently outperforms them in the field of WCE image analysis for automated lesion detection, providing higher classification results, up to 93.8% (accuracy), 95.2% (sensitivity), 92.4% (specificity) and 92.6% (precision). Validation cohort included |
| Klang *et al*[48], 2020 | Convolutional neural network (CNN) *vs* human reader | CD | Retrospective cohort, 49 CD patients | WCE images | Endoscopic Inflammation | Dataset included 17640 CE images from 49 patients: 7391 images with mucosal ulcers and 10249 images of normal mucosa. For randomly split images results, AUC was 0.99 with accuracies ranging from 95.4% to 96.7%. For individual patient-level experiments, the AUCs were 0.94-0.99. Validation cohort included |
| Ungaro *et al*[49], 2021 | Random survival forest. No comparator | Peds CD | Retrospective case-control, 265 peds CD patients | Protein biomarkers using a proximity extension assay (Olink Proteomics) | Penetrating and stricturing complications | A model with 5 protein markers predicted penetrating complications with an AUC of 0.79 (95%CI: 0.76-0.82) compared to 0.69 (95%CI: 0.66-0.72) for serologies and 0.74 (95%CI: 0.71-0.77) for clinical variables. A model with 4 protein markers predicted structuring complications with an AUC of 0.68 (95%CI: 0.65-0.71) compared to 0.62 (95%CI: 0.59-0.65) for serologies and 0.52 (95%CI: 0.50-0.55) for clinical variables. Validation cohort included |

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| Barash *et al*[50], 2021 | Ordinal CNN. No comparator | CD | Retrospective cohort, 49 CD patients | WCE images | Ulcer Severity Grading | The classification accuracy of the algorithm was 0.91 (95%CI: 0.867-0.954) for grade 1 *vs* grade 3 ulcers, 0.78 (95%CI: 0.716-0.844) for grade 2 *vs* grade 3, and 0.624 (95%CI: 0.547-0.701) for grade 1 *vs* grade 2. Validation cohort included |
| Lamash *et al*[51], 2019 | CNN *vs* semi-supervised and active learning models | CD | Retrospective cohort, 23 CD patients | Magnetic resonance imaging | Active Crohn's Disease | CNN exhibited Dice similarity coefficient of 75% ± 18%, 81% ± 8%, and 97% ± 2% for the lumen, wall, and background, respectively. The extracted markers of wall thickness at the location of min radius ($P$ = 0.0013) and the median value of relative contrast enhancement ($P$ = 0.0033) could differentiate active and nonactive disease segments. Other extracted markers could differentiate between segments with strictures and segments without strictures ($P$ < 0.05). Validation cohort included |
| Takenaka *et al*[52], 2020 | Deep neural networks *vs* human reader (endoscopist) | UC | Prospective cohort, 2012 UC patients | Colonoscopy images | Endoscopic inflammation | Deep neural network identified patients with endoscopic remission with 90.1% accuracy (95%CI: 89.2-90.9) and a kappa coefficient of 0.798 (95%CI: 0.780-0.814), using findings reported by endoscopists as the reference standard. Validation cohort included |
| Bossuyt *et al* [53], 2020 | Computer algorithm based on red density (RD) *vs* blinded central readers | UC | Prospective cohort, 29 UC patients, 6 healthy controls | Colonoscopy Images | Endoscopic and histologic inflammation | In the construction cohort, RD correlated with rhi ($r$ = 0.74, $P$ < 0.0001), Mayo endoscopic subscores ($r$ = 0.76, $P$ < 0.0001) and Endoscopic index of severity scores ($r$ = 0.74, $P$ < 0.0001). The RD sensitivity to change had a standardized effect size of 1.16. in the validation set, RD correlated with rhi ($r$ = 0.65, $P$ = 0.00002). Validation cohort included |
| Bhambhvani *et al*[54], 2021 | CNN *vs* human reader (endoscopist) | UC | Retrospective cohort, 777 UC patients | Colonoscopy images | Mayo Endoscopic Scores (MES) | The final model classified MES 3 disease with an AUC of 0.96, MES 2 disease with an AUC of 0.86, and MES 1 disease with an AUC 0.89. Overall accuracy was 77.2%. Across MES 1, 2, and 3, average specificity was 85.7%, average sensitivity was 72.4%, average PPV was 77.7%, and the average NPV was 87.0%. Validation cohort included |
| Ozawa *et al* [55], 2019 | CNN *vs* human reader (endoscopist) | UC | Retrospective cohort, 841 UC patients | Colonoscopy images | MES | The CNN-based CAD system showed a high level of performance with AUC of 0.86 and 0.98 to identify Mayo 0 and 0-1, respectively. The performance of the CNN was better for the rectum than for the right side and left side of the colon when identifying Mayo 0 (AUC = 0.92, 0.83, and 0.83, respectively). Validation cohort included |
| Bossuyt *et al*[56], 2021 | Automated CAD Algorithm *vs* human reader | UC | Prospective cohort, 48 UC patients | Colonoscopy images with confocal laser endomicroscopy | Histologic Remission | The current automated CAD algorithm detects histologic remission with a high performance (sensitivity of 0.79 and specificity of 0.90) compared with the UCEIS (sensitivity of 0.95 and specificity of 0.69) and MES (sensitivity of 0.98 and specificity of 0.61). No validation cohort included |
| Stidham *et al*[57], 2019 | CNN *vs* human reader | UC | Retrospective cohort, 3082 UC patients | Colonoscopy images | Endoscopy severity | The CNN was excellent for distinguishing endoscopic remission from moderate-to-severe disease with an AUC of 0.966 (95%CI: 0.967-0.972); a PPV of 0.87 (95%CI: 0.85-0.88) with a sensitivity of 83.0% (95%CI: 80.8-85.4) and specificity of96.0% (95%CI: 95.1-97.1); and NPV of 0.94 (95%CI: 0.93-0.95). No validation cohort included |
| Gottlieb *et al*[58], 2021 | Neural network *vs* human central reader | UC | Prospective cohort, 249 UC patients | Colonoscopy images | Endoscopy severity | The model's agreement metric was excellent, with a quadratic weighted kappa of 0.844 (95%CI: 0.787-0.901) for endoscopic Mayo Score and 0.855 (95%CI: 0.80-0.91) for UCEIS. No validation cohort included |

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve; NPV: Negative predictive value; PPV: Positive predictive value; qPCR: Quantitative real-time polymerase chain reaction; HR: Hazard ratio.

Table 3 summarizes studies included which applied AI in the prediction of therapy response and clinical outcomes in IBD. There were 9 studies that predicted therapy response, 2 studies that predicted presence of extraintestinal manifestations of IBD, 1 study predicting colonic neoplasia, and 1 study predicting post-surgical complications after colectomy. Data modalities included electronic health records ($n$ = 11), molecular

**Table 3 Artificial intelligence in prediction of therapy response and clinical outcomes in inflammatory bowel disease**

| Ref. | AI classifier *vs* comparator | IBD type | Study design and sample size | Modality | Outcomes | Study results/validation cohort |
|---|---|---|---|---|---|---|
| Waljee *et al*[59], 2018 | Random forest (RF). No comparator | CD/UC | Post-hoc analysis of prospective clinical trial, 594 CD patients | Veteran's Health Administration Electronic Health Record (EHR) | Outpatient corticosteroids prescribed for IBD and inpatient hospitalizations associated with a diagnosis of IBD | AUC for the RF longitudinal model was 0.85 [95% confidence interval (CI): 0.84–0.85]. AUC for the RF longitudinal model using previous hospitalization or steroid use was 0.87 (95%CI: 0.87-0.88). Validation cohort included |
| Uttam *et al*[60], 2019 | Support vector machines (SVM) *vs* nanoscale nuclear architecture mapping (NanoNAM) | CD/UC | Prospective cohort, 103 IBD patients | 3-dimensional NanoNAM of normal-appearing rectal biopsies | Colonic neoplasia | NanoNAM detects colonic neoplasia with an AUC of 0.87 ± 0.04, sensitivity of 0.81 ± 0.09, and specificity of 0.82 ± 0.07 in the independent validation set. Validation cohort included |
| Waljee *et al*[61], 2017 | RF. No comparator | CD/UC | Retrospective cohort, 1080 IBD patients | EHR, lab values | Remission and clinical outcomes with thiopurines | AUC for algorithm-predicted remission in the validation set was 0.79 *vs* 0.49 for 6-TGN. The mean number of clinical events per year in patients with sustained algorithm-predicted remission (APR) was 1.08 *vs* 3.95 in those that did not have sustained APR ($P < 1 \times 10^{-5}$). Validation cohort included |
| Popa *et al*[62], 2020 | Neural network model. No comparator | UC | Prospective cohort, 55 UC patients | Clinical and biological parameters and the endoscopic Mayo score | Disease activity after one year of anti-TNF treatment | The classifier achieved an excellent performance predicting the disease activity at one year with an accuracy of 90% and AUC 0.92 on the test set and an accuracy of 100% and an AUC of 1 on the validation set. Validation cohort included |
| Douglas *et al*[45], 2018 | RF. No comparator | Peds CD | Cross-sectional, 20 CD patients, 20 healthy controls | Shotgun metagenomics (MGS), 16S rRNA gene sequencing | Response to induction therapy | 16S genera were again the top dataset (accuracy = 77.8%; $P = 0.008$) for predicting response to therapy. MGS strain ($P = 0.029$), genus ($P = 0.013$), and KEGG pathway ($P = 0.018$) datasets could also classify patients according to therapy response with accuracy = 72.2% for all three. Validation cohort included |
| Waljee *et al*[63], 2010 | RF *vs* boosted trees, RuleFit | CD/UC | Cross-sectional, 774 IBD patients | EHR, lab values (thiopurine metabolites) | Response to thiopurine therapy | A RF algorithm using laboratory values and patient age differentiated clinical response from nonresponse in the model validation data set with an AUC of 0.856 (95%CI: 0.793-0.919). Validation cohort included |
| Menti *et al*[64], 2016 | Naïve bayes *vs* Bayesian additive regression trees *vs* Bayesian networks | CD/UC | Retrospective cohort, 152 CD patients | Genomic DNA, genetic polymorphism | Presence of extra-intestinal manifestations in IBD patients | Bayesian networks achieved accuracy of 82% when considering only clinical factors and 89% when considering also genetic information, outperforming the other techniques. Validation cohort included |
| Waljee *et al*[65], 2017 | RF *vs* baseline regression model | CD/UC | Retrospective cohort, 20368 IBD patients | EHR, lab values | Corticosteroid-free biologic remission with vedolizumab | The AUC for corticosteroid-free biologic remission at week 52 using baseline data was only 0.65 (95%CI: 0.53-0.77), but was 0.75 (95%CI: 0.64-0.86) with data through week 6 of vedolizumab. Validation cohort included |
| Morilla *et al*[66], 2019 | Deep neural networks. No comparator | UC | Retrospective cohort, 47 UC patients | Colonic microrna profiles | Responses to therapy | A deep neural network-based classifier identified 9 microRNAs plus 5 clinical factors, routinely recorded at time of hospital admission, that were associated with responses of patients to treatment. This panel discriminated responders to steroids from non-responders with 93% accuracy (AUC, 0.91). Three algorithms, based on microRNA levels, identified responders to infliximab *vs* non-responders (84% accuracy, AUC 0.82) and responders to cyclosporine *vs* non-responders (80% accuracy, AUC 0.79). Validation cohort included |
| Wang *et al*[67], 2020 | Back-propagation neural network (BPNN), SVM *vs* logistic regression | CD | Cross-sectional, 446 CD patients | EHR | Medication nonadherence to maintenance therapy | The average classification accuracy and AUC of the three models were 85.9% and 0.912 for BPNN, and 87.7% and 0.930 for SVM, respectively. Validation cohort included |
| Bottigliengo | Bayesian machine | CD/UC | Retrospective cohort, | EHR, genetic | Presence of extra-intestinal | BMLTs had an AUC of 0.50 for classifying the presence of extra-intestinal manifestations. Validation |

| | | | | | | |
|---|---|---|---|---|---|---|
| *et al*[68], 2019 | learning techniques (BMLTs) *vs* logistic regression | | 142 IBD patients | polymorphisms | manifestations in IBD patients | cohort included |
| Ghoshal *et al*[69], 2020 | Nonlinear artificial neural network (ANN) *vs* multivariate linear PCA | UC | Prospective cohort, 263 UC patients | EHR | Responses to therapy | The multilayer perceptron neural network was trained by back-propagation algorithm (10 networks retained out of 16 tested). The classification accuracy rate was 73% in correctly classifying response to medical treatment in UC patients. No validation cohort included |
| Sofo *et al*[70], 2020 | SVM leave-one-out cross-validation. No comparator | UC | Retrospective cohort, 32 UC patients | EHR | Post-surgical complications after colectomy | Evaluating only preoperative features, machine learning algorithms were able to predict minor postoperative complications with a high strike rate (84.3%), high sensitivity (87.5%) and high specificity (83.3%) during the testing phase. Validation cohort included |
| Kang *et al*[71], 2017 | ANN *vs* logistic regression | UC | Cross-sectional, 24 UC patients | Gene expression profiles | Response to anti-TNF | Balanced accuracy in cross validation test for predicting response to anti-TNF therapy in ulcerative colitis patient was 82%. Validation cohort included |
| Babic *et al*[72], 1997 | CART *vs* back propagation neural network (BPNN) | CD/UC | Cross-sectional, 200 IBD patients | EHR | Quality of life | Best reached classification accuracy did not exceed 80% in any case. Other classifiers namely, K-nearest-neighbor, learning vector quantization and BPNN confirmed that outcome. Validation cohort included |
| Dong *et al*[73], 2019 | RF, SVM, ANN *vs* logistic regression | CD | Retrospective cohort, 239 CD patients | EHR, laboratory tests | Crohn's related surgery | The results revealed that RF predictive model performed better than LR model in terms of accuracy (93.11% *vs* 91.15%), precision (53.42% *vs* 44.81%), F1 score (0.6016 *vs* 0.5763), TN rate (95.08% *vs* 92.00%), and the AUC (0.8926 *vs* 0.8809). The AUCs were excellent at 0.9864 in RF,0.9538 in LR, 0.8809 in DT, 0.9497 in SVM, and 0.9059 in ANN, respectively. Validation cohort included |
| Lerrigo *et al*[74], 2019 | Latent Dirichlet allocation, unsupervised machine learning algorithm. No comparator | CD/UC | Retrospective cohort, 28623 IBD patients | Online posts from the Crohn's and colitis foundation community forum | Impact of online community forums on well-being and their emotional content | 10702 (20.8%) posts were identified expressing: gratitude (40%), anxiety/fear (20.8%), empathy (18.2%), anger/frustration (13.4%), hope (13.2%), happiness (10.0%), sadness/depression (5.8%), shame/guilt (2.5%), and/or loneliness (2.5%). A common subtheme was the importance of fostering social support. No validation cohort included |

AI: Artificial intelligence; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AUC: Area under the curve; TNF: Tumor necrosis factor.

datasets ($n$ = 4), and histologic data ($n$ = 1). Waljee *et al*[59,65] and Popa *et al*[62] have previously applied RF classifiers to clinical data from electronic health records and laboratory values to predict response to various IBD therapies. In one study using data from a prospective clinical trial consisting of 594 CD patients[59], the AUC for a RF longitudinal model for predicting inpatient hospitalizations in IBD patients prescribed outpatient corticosteroids was 0.85 (95%CI: 0.84-0.85). Using a similar RF approach for predicting remission with thiopurine therapy in a prospective cohort of 55 UC patients yielded an AUC of 0.79[62]. Applying RF to data from a retrospective cohort of 20368 IBD patients with vedolizumab use yielded an AUC of 0.65 (95%CI: 0.53-0.77) for corticosteroid-free vedolizumab remission at week 52 using baseline data and an AUC of 0.75 (95%CI: 0.64-0.86) with data through week 6 of vedolizumab[65]. Molecular datasets have also been used to differentiate between responders and non-responders to various IBD therapies. For example, Morilla *et al*[66] used a deep neural network classifier to construct a predictive panel of colonic microRNAs for IBD therapies in a retrospective cohort of 47 UC patients. Their panel discriminated responders to steroids from non-responders with 93% accuracy (AUC, 0.91). In addition, three

algorithms, based on microRNA levels, identified responders to infliximab *vs* non-responders (84% accuracy, AUC 0.82) and responders to cyclosporine *vs* non-responders (80% accuracy, AUC 0.79). A more recent prospective cohort study of 55 UC patients by Popa *et al*[62] integrated clinical, laboratory, and endoscopic (Mayo scores) datasets using a neural network classifier to predict disease activity after one year of anti-tumor necrosis factor therapy in patients with UC. This classifier achieved an AUC of 0.92 for predicting the disease activity at one year on the test set and an AUC of 1.00 on the validation set. These studies suggest that AI classifiers may play a role in predicting clinical outcomes and response to specific therapies in patients with IBD. However, future clinical trials are needed to compare the efficacy of AI applications in IBD clinical management *vs* standard of care before incorporation into real life clinical practice.

Finally, AI algorithms have been previously applied to enhance the detection of colonic polyps[87] and distinguish among subtypes of neoplastic colorectal lesions[88] in the general population. Although patients with IBD who have extensive colitis have a significantly greater risk of colorectal cancer compared to the general population [89,90], there have been limited studies applying AI technologies to improve colorectal cancer surveillance or develop prediction risk models in patients with IBD. Most studies evaluating polyp detection have excluded IBD patients[91-93]. Our literature search yielded only one study applying AI for the detection of colonic neoplasia in IBD. Uttam *et al*[60] employed support SVM to analyze 3-dimensional nanoscale nuclear architecture mapping (NanoNAM) of normal-appearing rectal biopsies in a prospective cohort of 103 IBD patients. In their study, NanoNAM detected colonic neoplasia with an AUC of 0.87 ± 0.04, sensitivity of 0.81 ± 0.09, and specificity of 0.82 ± 0.07 in the independent validation set. Further studies should focus on determining the clinical utility of incorporating AI methods to enhance standard of cancer surveillance in patients with IBD such as chromoendoscopy[94] and to develop predictive models for risks of colorectal malignancy in IBD patient populations.

## CONCLUSION

In conclusion, our literature review has revealed that the applications of AI in IBD have significantly increased in recent years. Our review also highlighted that various AI classifiers may be applied to analyze and integrate large datasets ranging from clinical data from electronic health records, molecular data including gene expression and protein-based studies to a wide array of datasets consisting of endoscopic and histologic images. The application of AI has the potential to improve the accuracy and precision of predicting risk and diagnosis of IBD, assessing disease severity, and predicting outcomes with various IBD therapies. Currently, the application of AI methods in IBD has been limited to the research setting and has not yet been adopted in real life clinical practice. Furthermore, studies applying AI in the context of colorectal cancer surveillance or prediction in IBD are much needed. Given the current status of the field of AI in IBD, future directions should include: (1) Prospective validation of AI applications in IBD in independent cohorts as there is a risk of bias from internal training cohorts and potential limitations with generalizability; (2) Standardization of AI methods and comparative studies evaluating effect of heterogeneity from using different types of datasets on outcomes of interest; (3) Randomized controlled trials to determine whether application of AI in the clinical management of IBD improves clinical outcomes and could be translated into clinical practice; and (4) Randomized controlled trials to determine whether application of AI leads to greater clinical efficacy and cost-effectiveness compared to standard of care in IBD.

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MINIREVIEWS

# Post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition as harbingers of pancreatic cancer

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## Abstract

Progress in the fight against pancreatic cancer has been hampered by many factors. One of them is the inability to detect the disease early in overwhelming majority of patients. The present paper outlines a novel way in which progress could be accelerated. This includes a focus on two harbingers—post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition—that converge at affecting the tumor macroenvironment and microenvironment specifically in the pancreas, not other organs. The two entities have the potential to be incorporated into future screening strategies with a view to early detecting of pancreatic cancer.

**Key Words:** Pancreatic cancer; Post-pancreatitis diabetes mellitus; Intra-pancreatic fat; Pancreatitis; Early detection; Screening



**Core Tip:** Identification of harbingers of pancreatic cancer that are specifically related to the pancreas is necessary to enable cost-effective and achievement-appropriate screening for this disease. Post-pancreatitis diabetes mellitus and excess intra-pancreatic fat deposition are positioned well to serve the purpose.

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## INTRODUCTION

Pancreatic cancer incidence rates are on the rise since the 1990s[1]. Given that around 90% of pancreatic cancer is unresectable at the time of diagnosis, early detection of pancreatic cancer is critical with a view to lessening the burden of this disease[2]. The development of an accurate test for early detection of sporadic pancreatic cancer would considerably improve the survival of these patients[3]. However, while more than 2500 putative biomarkers (genomics, transcriptomics, proteomics) were found to be overexpressed at the messenger RNA or protein level in pancreatic cancer[4], translating biomarker discoveries into clinical applications has been a litany of failures in the setting of pancreatic cancer. Identification of harbingers of sporadic pancreatic cancer and development of screening policies based on them is another avenue towards lessening the burden of this disease. New-onset diabetes and obesity became recognized as one of the most prominent risk factors for pancreatic cancer[5-7]. The present review focuses on how that knowledge has crystallized over the past 5 years or so to consider post-pancreatitis diabetes mellitus (PPDM) and excess intra-pancreatic fat deposition (IPFD) as the specific harbingers of pancreatic cancer that are superior to general risk factors such as new-onset diabetes mellitus and obesity.

## PERSISTENT HYPERGLYCEMIA

A 2011 meta-analysis of 35 cohort studies showed that people with prevalent diabetes had a 1.9-times higher risk for pancreatic cancer as compared with those without diabetes (Figure 1)[8]. Later, a 2018 study from the Mayo clinic demonstrated that not any diabetes but only incident diabetes holds promise as a harbinger of pancreatic cancer[9]. A 60-mo temporal fasting plasma glucose profile was constructed for patients diagnosed with pancreatic cancer (as well as matched controls). The authors showed that hyperglycemia first occurred 30-36 mo prior to pancreatic cancer diagnosis and reached the diabetes threshold 6-12 mo prior to cancer diagnosis. Moreover, fasting plasma glucose concentrations increased with tumor volume, with the smallest tumor volume associated with hyperglycemia being 1.1-2.0 mL (which is considerably smaller than the average tumor volume of 11.5 mL at diagnosis of pancreatic cancer)[9]. In theory, diagnosing pancreatic cancer when it is that small could markedly increase cure rates and long-term survival. In practice, however, hyperglycemia alone cannot be implemented as a cost-effective screening strategy because pancreatic cancer is rare whereas hyperglycemia is very common. Further, hyperglycemia in the context of pancreatic cancer represents a paraneoplastic syndrome and therefore is not specific. For example, data from two large prospective cohorts (494078 person-years of follow-up) in the United States published in 2020 confirmed that incident diabetes is a significant risk factor for pancreatic cancer (adjusted hazard ratio 2.07; 95% confidence interval 1.70 to 2.52)[10]. However, the study also showed that incident diabetes is a significant risk factor for cancer in 7 other organs (breast, large intestine, endome-trium, esophagus, liver, lung, and thyroid). Combining incident diabetes with weight loss was shown to increase the ability to predict the occurrence of pancreatic cancer in a 2018 retrospective study[11]. However, weight loss is another non-specific symptom and therefore is unlikely to be much more useful in determination of pancreatic cancer risk (as compared with cancer in the 7 organs mentioned above).

It is conceivable that accurate determination of pancreatic cancer risk among people with persistent hyperglycemia can only be achieved when factors related specifically to the pancreas are considered. One such factor is inflammation of the pancreas prior to new-onset diabetes (*i.e.*, PPDM). PPDM is a sub-type of diabetes of the exocrine pancreas and is caused by acute pancreatitis in four out of five people and chronic pancreatitis in one out of five people[12]. Its epidemiology, risk factors, pathogenesis, and management were comprehensively reviewed elsewhere[12]. A large 2020 cohort study by Cho *et al*[13] compared the risks of developing pancreatic cancer in PPDM *vs* type 2 diabetes mellitus without history of pancreatitis and showed that PPDM was associated with a 7-times significantly higher risk for pancreatic cancer (adjusted hazard ratio 6.94; 95% confidence interval 4.09 to 11.77). This held true after adjustment for age, sex, ethnicity, social deprivation index, alcohol abuse, tobacco smoking, history of gallstones, cholecystectomy, and Charlson comorbidity index. When a 12-mo lag period between diabetes diagnosis and pancreatic cancer diagnosis was introduced (to minimize the possibility of reverse causality), the results did not change materially (adjusted hazard ratio 7.93; 95% confidence interval 3.53 to 17.81).

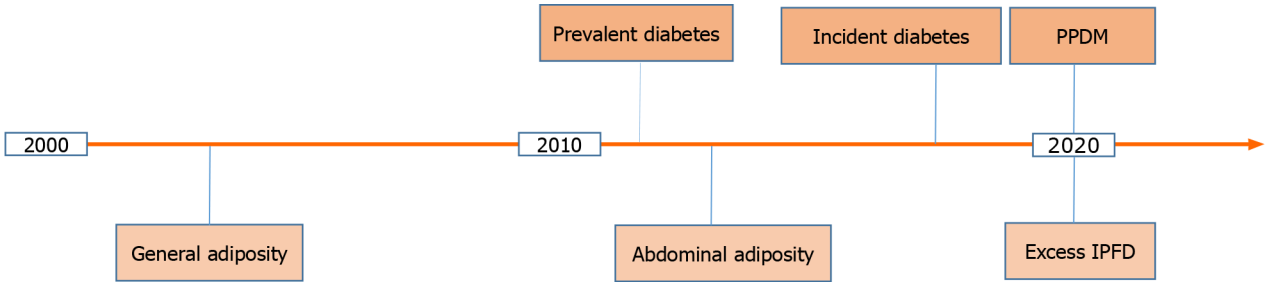


**Figure 1 A timeline of the major developments in regards to persistent hyperglycemia and excess body fat as harbingers of pancreatic cancer in the 21st century to date.** IPFD: Intra-pancreatic fat deposition; PPDM: Post-pancreatitis diabetes mellitus.

Also, people with history of pancreatitis (without diabetes mellitus) had a 4.8-times significantly higher risk of pancreatic cancer (95% confidence interval 3.38 to 6.99) than those with type 2 diabetes mellitus without history of pancreatitis[13]. This suggests that diabetes mellitus without history of pancreatitis is not a major risk factor for pancreatic cancer; rather it is pancreatitis that is a major risk factor for pancreatic cancer in individuals with diabetes. Moreover, the study showed that an attack of pancreatitis in individuals with diabetes had a differential effect on the subsequent risk of pancreatic cancer depending on whether it occurred before or after diabetes. Specifically, it found that people with PPDM had a 2.3-times significantly higher risk of pancreatic cancer (95% confidence interval 1.12 to 4.93) than those with type 2 diabetes mellitus that precedes pancreatitis, after adjustment for the above-mentioned covariates[13]. This suggests that the increased risk of pancreatic cancer in individuals with PPDM is not due to merely the effect of pancreatitis as a comorbidity in individuals with type 2 diabetes mellitus but rather pancreatitis exerts an effect beyond being a comorbidity in individuals with PPDM.

The 2018 Mayo clinic study[9] and the 2020 COSMOS study[13] are highly complemen-tary in nature, paving the way to identification of population at high risk of pancreatic cancer within a cohort of people with diabetes, which has the potential to enrich the cohort for pancreatic cancer. The 3-year incidence of pancreatic cancer in the Mayo clinic study was 1.0% among individuals with diabetes, which is in line with the 0.7% estimate in individuals with diabetes in the entire cohort of the COSMOS study. The Mayo clinic developed a model using the data of 1516 individuals with first diagnosis of diabetes (based on fasting blood glucose and/or estimated average glucose) and the incidence of pancreatic cancer increased to 3.6% after applying the model[11]. The model requires five variables: age at first diagnosis of diabetes, blood glucose levels at two time points (approximately 12 mo prior to and at first diagnosis of diabetes), and weight at two time points (approximately 12 mo prior to and at first diagnosis of diabetes). The COMSOS study of 139843 individuals offered a complementary non-overlapping approach, in which the consideration of history of pancreatitis prior to first diagnosis of diabetes (regardless of changes in glycemia and weight prior to diabetes) enabled the enrichment of the cohort of people with diabetes for pancreatic cancer to the extent the Mayo clinic study did (from 0.7% to 3.1% in the COSMOS study as compared with from 1.0% to 3.6% in the Mayo clinic study)[13]. Interestingly, the COSMOS study found that resected pancreatic cancer yielded the highest risk (hazard ratio 16.2) in individuals with PPDM[13]. This likely reflects the higher likelihood of detection of pancreatic cancer at earlier stages in individuals with PPDM *vs* type 2 diabetes mellitus, which may be attributable to the fact that individuals with PPDM are more likely to undergo more intensive work-up during hospitalization for pancreatitis (*e.g.*, earlier abdominal imaging and carbohydrate antigen 19-9, possibly resulting in a lead time) and are more closely monitored after hospital discharge. This is not dissimilar to the notion of 'incidentaloma'—incidental abnormal finding from imaging test. Based on the above findings, it is reasonable to suggest that taking into account history of pancreatitis (in addition to age at diabetes diagnosis and changes in glycemia and body composition prior to diabetes) will further enrich cohorts of people with diabetes for pancreatic cancer. Purposely-designed studies are warranted to operationalize the combined approach. But, in principal, it could be applied to all middle-aged and older adults after an attack of pancreatitis who develop new-onset diabetes and unintentional changes in body composition during follow-up. This might ultimately make screening for pancreatic cancer cost-effective and achievement-appropriate.

## EXCESS BODY FAT

In a 2003 prospective cohort study of more than 900000 adults, the relative risk of pancreatic cancer for people with morbid obesity (body mass index > 40 kg/m$^2$) was 2.76 (95% confidence interval 1.74 to 4.36) for women and 2.61 (95% confidence interval 1.30 to 5.40) for men (Figure 1)[14]. A 2009 prospective cohort study of more than 450416 adults estimated that general overweight or obesity (body mass index ≥ 25 kg/m$^2$) explained 8% of the population attributable risk for pancreatic cancer, which made it the second largest population attributable risk (following tobacco smoking) among all the modifiable factors studied[15]. Later, visceral adiposity (as evidenced by waist circumference) became acknowledged as a more accurate measure of excess body fat (Figure 1). Several prospective studies showed a significant association between risk of pancreatic cancer and visceral adiposity. These studies (encompassing 787356 adults) were meta-analyzed in 2012 and the risk of pancreatic cancer was estimated to increase 1.1-times (95% confidence interval 1.05 to 1.18) with every 10-cm increase in waist circumference[16]. Based on the best available evidence in regards to both body mass index and waist circumference, the World Cancer Research Fund and the American Institute for Cancer Research concluded that the association between excess adiposity and pancreatic cancer is causal[17]. However, the causality was also postulated in relation to excess adiposity and cancer in several other organs (esophagus, liver, colorectum, breast, endometrium, kidney). Given that both general adiposity and abdominal adiposity have a low specificity, these are not useful specifically for the purpose of early detection of pancreatic cancer.

More recently, local fat contained within the pancreas—termed IPFD—has emerged as an early specific factor contributing to the formation of pancreatic tumorigenesis (Figure 1). The relationship between IPFD and pancreatic cancer or premalignant lesions had been investigated in several studies that were systematically reviewed in a 2020 systematic review and meta-analysis by Sreedhar *et al*[18]. A total of 13 retrospective studies (encompassing 2178 individuals) were included. The pooled prevalence of fatty pancreas disease in individuals with pancreatic cancer was 52% (95% confidence interval 38 to 66%). Further, there was a 2.8-times higher prevalence of fatty pancreas disease among individuals with pancreatic cancer or pre-malignant lesions compared with controls (risk ratio 2.78; 95% confidence interval 1.56 to 4.94)[18]. High IPFD was also associated with dissemination and increased mortality of the disease in two single-center studies[19,20]. Besides, there was an evidence of a consistent association between the presence of pancreatic pre-malignant lesions and high IPFD, independent of fatty liver disease, abdominal adiposity, and general adiposity. In particular, one study showed a significantly increased IPFD in individuals with intraductal papillary mucinous neoplasm ($n$ = 85), as compared with age-, sex-, and diabetes status-matched individuals with no pancreatic cyst ($n$ = 85)[21]. Taking into account that two types of pancreatic cancer can develop in individuals with intraductal papillary mucinous neoplasm (invasive carcinoma within the index lesion and concomitant pancreatic ductal adenocarcinoma arising at a site other than intraductal papillary mucinous neoplasm) and taking into account that progression to high-grade dysplasia within the index lesion is relatively easy to detect and follow up[22,23], an increased IPFD during follow-up could be particularly helpful in identifying individuals with intraductal papillary mucinous neoplasm who harbor concomitant pancreatic ductal adenocarcinoma.

IPFD was also investigated in the setting of pancreatitis—a major risk factor for pancreatic cancer[13,24-27]. A cross-sectional study by Stuart *et al*[28] investigated 119 individuals after an attack of pancreatitis and 38 healthy volunteers. It found that IPFD (determined with the use of chemical shift-encoded magnetic resonance imaging) was significantly greater in individuals after an attack of pancreatitis (both acute and chronic) than healthy volunteers, in both crude analysis and after adjustment for age, sex, ethnicity, visceral-to-subcutaneous fat volume ratio, glycated hemoglobin, triglycerides. Notably, two other common ectopic fat phenotypes—liver fat and skeletal muscle fat deposition—did not differ significantly between the groups[28]. Several other cross-sectional studies showed that excess IPFD is associated with worse outcomes during hospitalization for acute pancreatitis[29-31]. Individuals with chronic pancreatitis alone ($n$ = 58) had a significantly greater IPFD in comparison with controls ($n$ = 60) in a cross-sectional study from the United States (determined with the use of chemical shift-encoded magnetic resonance imaging)[32]. Also, the severity of pancreatic ductal changes (based on the Cambridge classification) in individuals with chronic pancreatitis was not associated with IPFD[32]. It is worth noting that the study groups were compared in crude analysis only in that study, despite the fact that there were significant differences between the groups in terms of age, body composition,

alcohol consumption, and tobacco smoking. An earlier study from the United States found that individuals with chronic pancreatitis alone ($n$ = 35) had a significantly greater IPFD in comparison with controls ($n$ = 50) in a post-hoc analysis constrained to non-obese people only (body mass index < 30 kg/m$^2$)[33]. A longitudinal study from Japan sought to investigate the temporal relationship between IPFD and chronic pancreatitis[34]. A total of 9933 individuals without pancreatitis were examined in 2008 and followed up for 4 years as part of their medical check-up. The presence of fatty pancreas disease at baseline was associated with a 3.9-times higher risk of incident pancreatitis during follow-up (odds ratio 3.9; 95% confidence interval 2.0 to 7.7), after adjustment for age, sex, body mass index, glycated hemoglobin, systolic blood pressure, alcohol abuse, tobacco smoking, and other covariates[34]. However, it is worth noting that transabdominal ultrasound was used in this study, which is suboptimal for diagnosing of both chronic pancreatitis and fatty pancreas disease.

Beyond people with pancreatic premalignant lesions or history of pancreatitis, it is tempting to speculate that people with incidentally found fatty pancreas disease could benefit from a regular follow-up with a view to early detecting of pancreatic cancer. However, given that fatty pancreas disease is very common in the general population (prevalence 16.1%; 95% confidence interval 13.3 to 18.8)[35] and taking into account that the state-of-the-art sequential assessment of the pancreas (*i.e.*, the use of magnetic resonance imaging) is costly[36], screening of unselected people with fatty pancreas disease for pancreatic cancer is unlikely to reach current cost-effectiveness standards. However, it is envisaged that future studies will identify a subgroup of people with fatty pancreas disease in the general population that is at high risk for sporadic pancreatic cancer.

## CONCLUSION

The complex nature and the relative rarity of pancreatic cancer make it challenging to implement screening in people with no family history of the disease[37,38]. In fact, a 2019 evidence-based report by the United States Preventive Services Task Force deemed screening for pancreatic cancer in asymptomatic adults not to be cost-effective[39]. However, to date, the cost-effectiveness of only conventional non-specific risk factors has been considered. A 2021 microsimulation screening analysis model investigated the impact of relevant uncertainties on the effectiveness of pancreatic cancer screening and showed that test specificity had higher influence than sensitivity[40]. Growing evidence compels a consideration of middle-aged and older adults with PPDM and/or incidentally found fatty pancreas disease as specific populations at very high risk of developing pancreatic cancer. Comprehensive understanding of the intricate relationship between PPDM and IPFD will offer actionable insights into early detection of pancreatic cancer.

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*MINIREVIEWS*

# *BRCA* mutated pancreatic cancer: A change is coming

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## Abstract

Pancreatic cancer remains a leading cause of cancer-related death with few available therapies for advanced disease. Recently, patients with germline *BRCA* mutations have received increased attention due to advances in the management of *BRCA* mutated ovarian and breast tumors. Germline *BRCA* mutations significantly increase risk of developing pancreatic cancer and can be found in up to 8% of patients with sporadic pancreatic cancer. In patients with germline *BRCA* mutations, platinum-based chemotherapies and poly (ADP-ribose) polymerase inhibitors are effective treatment options which may offer survival benefits. This review will focus on the molecular biology, epidemiology, and management of *BRCA*-mutated pancreatic cancer. Further-more, we will discuss future directions for this area of research and promising active areas of research.

**Key Words:** Pancreatic cancer; Systemic therapy; Platinum chemotherapy; *BRCA*; Deoxyribonucleic acid repair; Poly (ADP-ribose) polymerase inhibitors



**Core Tip:** Recent advances in the field of *BRCA*-mutated pancreatic cancer suggest that these patients benefit from platinum-based chemotherapy regimens. In light of new findings from the Pancreas Cancer Olaparib Ongoing trial, patients with germline *BRCA* mutations may benefit from maintenance treatment with olaparib, a Poly (ADP-ribose) polymerase inhibitors following response to platinum-based chemotherapy. Based on these important findings, all pancreatic cancer patients should be offered early access to genetic screening in order to identify patients who will benefit from these therapies.

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## INTRODUCTION

Pancreatic cancer (PC) remains one of the most aggressive malignancies, with a 5-year survival rate of 8%[1,2]. Incidence of PC has increased over the past 4 decades, making it a leading cause of cancer-related mortality in North America[1-3]. The vast majority of pancreatic cancers are ductal adenocarcinomas (PDAC) of the exocrine pancreatic glands, occurring most commonly in the head of the pancreas[4]. Most cases of PDAC are considered sporadic, however 5%-10% are estimated to be familial with patients having a family history of PDAC[5]. Several genetic syndromes are known to cause familial PDAC including mutations of deoxyribonucleic acid (DNA) mismatch repair genes (Lynch syndrome), *BRCA1* and *BRCA2* (hereditary breast cancer syndrome); however, in the vast majority of cases a genetic cause cannot be identified[5-7].

Currently, the only potentially curative treatment for PC is surgical resection which is only possible in the early stages of the disease (locoregional) and highly dependent on the degree of invasion of surrounding critical structures such as vessels and bile ducts. Unfortunately, only 15%-20% of PDAC cases are considered resectable, and of these, over 75% will have recurrence within 5 years of their resection[4]. Recent data suggests that in patients with good performance status, treatment with a combination regimen of fluorouracil, oxaliplatin, leucovorin and irinotecan (FOLFIRINOX) is the optimal adjuvant therapy following resection[8]. Because early stage PC is usually asymptomatic, the vast majority of patients present with either locally advanced (involvement of local vasculature) or metastatic disease[4]. In these patients chemotherapy and occasionally radiotherapy form the backbone of treatment and are used to relieve symptoms and modestly prolong life.

In the advanced setting of disease, the two standard of care palliative chemotherapy options include gemcitabine plus albumin-bound paclitaxel (nab-paclitaxel) and FOLFIRINOX. In the first-line setting, both have been shown to prolong overall survival (OS) relative to gemcitabine monotherapy in prospective, randomized clinical trials[9,10]. Even with these treatments, 2-year survival remains at 10% and median OS ranges from 8-11 mo[4].

Recent genomic evidence suggests that PDAC is a genetically heterogenous disease with different molecular subtypes, potentially explaining the failure of many novel therapies when trialled in unselected populations[11,12]. Currently, efforts are ongoing to identify select PDAC patient populations who would benefit from targeted therapies. A patient group which has garnered much interest are those with mutations of *BRCA1* and *BRCA2*. These genes are important players in the homologous DNA repair (HR) pathway and mutations of both genes are strong risk factors for the development of several cancers including, breast, ovarian, prostate and pancreatic cancer[13,14]. Importantly, *BRCA* mutations also have implications for treatment as they may increase tumor susceptibility to both DNA-damaging chemotherapies such as platinum chemotherapy (PtCh), as well as poly (ADP-ribose) polymerase (PARP) inhibitors in breast and ovarian cancers. More recently, work has been done to determine if these clinical features translate to BRCA-mutated pancreatic cancer. This review will discuss the biology, epidemiology and clinical implications of *BRCA* mutations in PDAC, and will discuss future directions for this area of research.

## MOLECULAR BIOLOGY OF HOMOLOGOUS REPAIR

Several reviews have previously described the biology of the HR system and the specific roles of *BRCA1/2*[15,16]. Briefly, DNA damage can occur as either a single-stranded DNA break (SSB) or double-stranded DNA break (DSB). HR along with non-homologous end joining (NHEJ) are the two major pathways that respond to DSB. HR has the highest fidelity and precision of the DSB repair pathways, therefore defects in this pathway (homologous repair deficiency, HRD) lead to error-prone repair and genomic instability, increasing cancer risk. Important proteins in the HR system include BRCA1, BRCA2, PALB2, ATM and RAD51[15]. Following DSB, BRCA1

negatively regulates factors involved in the NHEJ pathway (53BP1) and promotes end resection, an important first step in the HR pathway. BRCA1 directly interacts with PALB2 to bind BRCA2 which facilitates formation of RAD51 filaments later in the pathway[15]. RAD51 filament form along ssDNA created earlier by BRCA1-mediated end resection, allowing formation of homologous DNA and repair of the DSB (Figure 1)[15]. Notably, other proteins involved in the HR pathways such as PALB2 and ATM are also mutated in PC, highlighting the importance of HR pathway integrity in determining PDAC risk[11,17].

While *BRCA* mutations confer increased cancer risk, emerging evidence suggests they also may be important markers for personalized medicine. *In vitro* and *in vivo* evidence suggests that both platinum-based chemotherapies and PARP inhibitors are more effective in patients harboring *BRCA* mutations[11].

## EPIDEMIOLOGY AND DIAGNOSIS OF *BRCA*-MUTATED PDAC

### *Incidence of pathogenic BRCA mutations in sporadic and familial PDAC*

Mutations of the *BRCA1* and *BRCA2* genes were first identified as breast and ovarian cancer risk factors in the mid-1990s during studies aimed at characterizing the genes responsible for familial clustering of breast and ovarian cancers[18,19]. Early studies by the Breast Cancer Linkage Consortium identified a 2.3-fold and 3.5-fold increased risk of PC in carriers of *BRCA1* and *BRCA2* gene mutations, respectively[13,14]. In the general population, germline *BRCA* mutations occur at a rate between 1/300 and 1/800[20]. However, incidence varies based on population as certain ethnic groups harbor founder mutations, increasing the incidence of *BRCA* mutations in these subgroups. The strongest example of the founder effect in *BRCA* is the Ashkenazi Jewish (AJ) population, where the presence of 3 founder mutations have increased rates of *BRCA* mutation to 1/40[21]. Other groups with founder *BRCA* mutations who are therefore at increased risk include Dutch, Norwegian and French-Canadian populations[22].

Among unselected PC patient cohorts, multiple studies have aimed to estimate the incidence of germline pathogenic *BRCA* mutations. Prevalence estimates ranged from 0.7%-5.7% for *BRCA2* and 0.3%-2.3% for BRCA1 (Summarized in Table 1)[6,23-26]. Notably, the cohorts in these studies varied widely based on several factors which could influence estimates of prevalence, including, number of AJ PC patients included, the number of patients with family histories of cancer, and median patient age[23]. For example, in AJ PDAC patients, studies have found that up to 19% of patients harbour germline *BRCA* mutations[23,27,28].

In familial PC, *BRCA* mutations, especially *BRCA2* are also at increased frequency. In the case of *BRCA2* mutations, studies have found germline mutations in 3.7%-19% of patients with strong familial histories of PDAC[29-32]. This range in estimates is likely a result of different criteria for familial pancreatic cancer (FPC), and different studies methodologies. Studies finding higher rates of *BRCA2* mutation tended to have smaller sample sizes and included patients with three or more first- or second-degree relatives with PC, therefore included higher risk patients. Conversely, more recent studies have included larger sample sizes of patients, who met the more moderate FPC case definition (two first- or second- degree relatives with PC), finding more conservative estimates of prevalence (3.7% and 6%)[31,32]. Therefore, in patients with a stronger family history of PC, *BRCA* carrier status is more likely. The incidence of *BRCA1* mutations in FPC has not been studied as well as *BRCA2*, however a recent study by Zhen *et al*[31] found that germline *BRCA1* mutations were present in 1.2% of patients with FPC.

### *Diagnosis of BRCA-mutated PDAC and screening guidelines*

While the identification of patients carrying *BRCA* mutations has been important in determining cancer risk, the discovery of personalized medicine options for this population has increased the clinical importance of identifying *BRCA* carriers. Genetic testing guidelines vary by region however, are primarily based on cancer phenotype which includes family history of breast, ovarian, prostate and pancreatic cancer, AJ ancestry and clinical presentation. Recently, genetic testing guidelines are being increasingly questioned as evidence accumulates to suggest that they would miss a large proportion of patients harboring *BRCA* mutations who may benefit from PARP inhibitors or platinum chemotherapies. In 2007, a Norwegian study tested breast and ovarian cancer patients for germline mutations in *BRCA1* and *BRCA2* and identified that 50% of patients with germline *BRCA* mutations do not have family histories of

**Table 1 Summary of studies of incidence of germline *BRCA* mutations in unselected pancreatic cancer cohorts**

| Ref. | Year | Population | Cohort size (Number AJ) | Germline *BRCA1* pathogenic mutation incidence (%) | Germline *BRCA2* pathogenic mutation incidence (%) | Combined germline *BRCA* mutation Incidence |
|---|---|---|---|---|---|---|
| Holter *et al*[23] | 2015 | North American | 306 (33) | 1.0% | 3.6% | 4.6% |
| Brand *et al*[24] | 2018 | North American | 298 (26) | 1.3% | 1.3% | 2.6% |
| Mizukami *et al*[25] | 2020 | Japanese | 1005 (-) | 1.7% | 2.5% | 4.2% |
| Grant *et al*[6] | 2015 | North American | 290 (13) | 0.3% | 0.7% | 1% |
| Lowery *et al*[26] | 2018 | North American | 615 (111) | 2.3% | 5.7% | 8% |

AJ: Ashkenazi Jewish; *BRCA:* Breast cancer susceptibility gene.

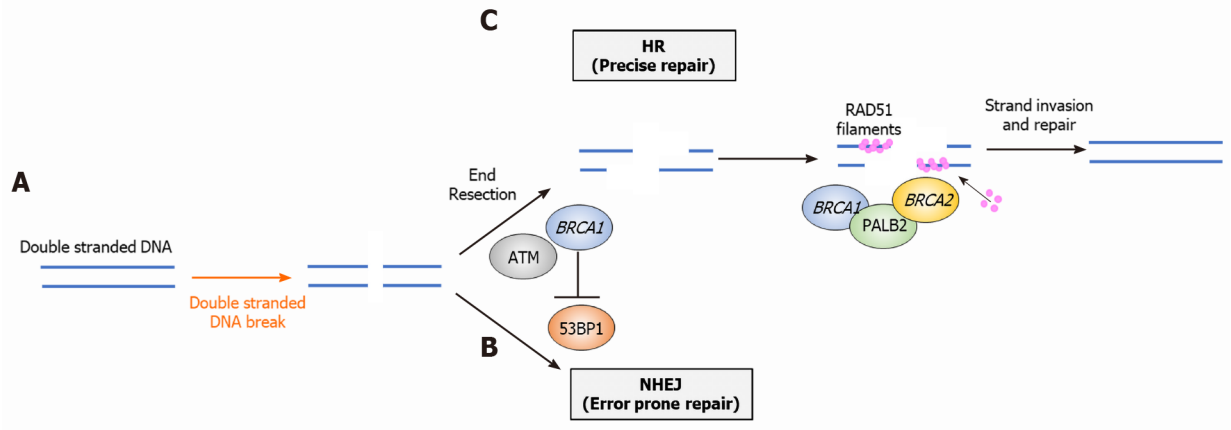


**Figure 1 Overview of the homologous repair pathway and roles of key proteins.** A: Following double strand break, BRCA 1 binds to the site of damage, mediating end resection and initiating homologous repair. This prevents repair *via* non-homologous end joining; B: *BRCA1* binds with PALB2 and *BRCA2* which facilitates assembly of RAD51 filaments; and C: RAD51 filaments form along ssDNA, subsequently leading to strand invasion and repair. DSB: Double strand break; HR: Homologous repair; NHEJ: Non-homologous end joining; BRCA: Breast cancer susceptibility gene.

*BRCA*-associated cancers[33]. Since then, multiple studies in different populations including patients with PDAC have confirmed these findings, showing poor associations between presence of *BRCA* mutations and expected family histories [23,34-38]. Furthermore, a recent study using data from 23&Me, a direct-to-consumer genetic test identified that 20% of carriers of the AJ founder variants don't identify as AJ, and therefore would be excluded from screening criteria that include AJ ancestry[39]. They also found that of 393 *BRCA* mutation carriers with available data on family cancer history, 44% had no family history of *BRCA*-associated cancers, and therefore, given a diagnosis of PDAC, would not meet screening requirements. The recent IMPACT trial by the Memorial Sloan-Kettering Cancer Centre provided strong evidence in favour of increased testing access. Investigators tested 1040 patients (176 PDAC) with advanced cancer and identified germline mutations in 21.5% of the PDAC patients. Notably, they found that across all cancers, 55% of clinically actionable mutations would not have been detected under current phenotype-based screening guidelines[40]. Together, this evidence strongly supports calls for increased access to genetic testing for PC patients. In early 2020, the National Comprehensive Cancer Network updated their recommenda-tions to suggest universal genetic testing for all PC patients as early as possible due to the rapid progression of the disease, and potential for early personalized therapy[41].

## CLINICAL FEATURES OF *BRCA*-MUTATED PDAC AND PROGNOSTIC IMPLICATIONS

While the ability of *BRCA* mutations to increase risk of PDAC is well established, their impact on the clinical features of the disease is less clear. Multiple cohort studies have shown in PDAC patients with germline mutations including *BRCA1, BRCA2, PALB2, CDKN2A* and *ATM*, are diagnosed earlier with PDAC than PDAC patients without germline mutations[31,42]. Conversely, a 2009 study comparing Jewish PDAC patients with and without germline *BRCA* mutations found no significant differences between age at diagnosis or any other clinicopathologic feature studied[28]. From a prognostic perspective, studies have shown mixed results. The largest cohort study to date including 71 *BRCA*-positive PDAC patients found a median OS of 14 mo for the whole cohort and 12 mo for patients with stage 3/4 disease. At time of publication, the median OS for early stage disease had not been reached as 52% of patients were still alive at 60 mo[43]. These findings suggest that *BRCA*-mutated PDAC patients may have a considerably better prognosis than the general PDAC population. On the contrary, more recent case-control studies by Blair *et al*[44] compared PDAC patients with *BRCA1* and *BRCA2* mutations to age-matched controls and showed that both OS and disease-free survival (DFS) were lower in carriers than controls. Another case-control study comparing *BRCA* mutation-positive, early-stage PDAC patients undergoing surgical resection to age-matched *BRCA*-wildtype controls found no significant differences in median OS or DFS between the groups and concluded that *BRCA* mutations were not prognostic in early PDAC[45]. Authors have suggested that early findings of improved prognosis in this population may have been a result of ascertainment bias as patients surviving longer were more likely to receive genetic testing and participate in the study. Another factor that may lead to improved prognosis in this patient population is increased susceptibility to treatments such as PtCh. Most recently, a study using data from the Know Your Tumor program aimed to assess whether mutations of HRD and other DNA-damage response (DDR) genes conferred a survival benefit or whether observed benefits were a result of increased PtCh-sensitivity[46]. The authors found that patients with advanced PDAC and HR/DDR mutations had improved survival but only if treated with PtCh. In PtCh-naïve patients, there was no survival benefit in this patient population[46].

Overall, identifying clinical differences between *BRCA*-mutated PDAC and wildtype PDAC has been difficult due to the relative rarity of these patients. Furthermore, the increasing use of personalized therapies (PARP inhibitors and platinum chemotherapy) in this population will make determining the prognostic implications of *BRCA* mutations more challenging.

## MANAGEMENT OF BRCA-MUTATED PDAC: SYSTEMIC THERAPY

### *Platinum chemotherapy*

While both FOLFIRINOX and gemcitabine/nab-paclitaxel chemotherapy regimens are more effective than gemcitabine monotherapy, there is yet to be a comparative randomized clinical trial to provide data on which regimen is more effective. In the locally advanced setting, a recent case series of 485 consecutive patients suggested that FOLFIRINOX was associated with a higher response rate (19% *vs* 6%, $P = 0.001$), however OS was not different with either treatment[47]. Retrospective studies in metastatic PDAC are inconclusive, with some studies reporting survival improvement on FOLFIRINOX while others report no difference between the two regimens[47,48]. Given the increased toxicity associated with FOLFIRINOX and potential survival benefits, identifying subsets of patients who are more likely to benefit from this regimen will be an important advancement in PC management.

The HRD phenotype of *BRCA*-mutated cancers appears to render them more sensitive to chemotherapies that induce DNA damage, such as PtCh. Early studies found that cells lacking *BRCA1* are more sensitive to treatment with cisplatin[49]. In the presence of HRD, these cells are unable to appropriately repair the DNA damage, leading to genomic instability and cell death[50]. Clinical studies in breast cancer have found that platinum-chemotherapy improves objective response rates (ORRs) for metastatic breast cancer patients only in *BRCA*-mutated cancers. Based on genomic studies in PDAC, it appears that tumors with *BRCA*-mutations have "unstable" molecular phenotypes and are more likely to be sensitive to genotoxic therapies such as PtCh[11].

In PC, several large retrospective studies have investigated the efficacy of PtCh such as FOLFIRINOX in patients with *BRCA* mutations or other genetic mutations leading to HRD (Table 2). To date, the largest cohort study was conducted by Golan *et al*[43] This multi-institution cohort study included 71 PC patients with germline *BRCA* mutations and found that among patients with advanced PDAC, OS was significantly longer in patients treated with PtCh (22 *vs* 9 mo). Since this study, several other retrospective cohort studies have reported improved outcomes [ORR, progression free survival (PFS)] in patients with germline mutations to HR-related genes who were treated with PtCh in both resectable and non-resectable PDAC[35,44,51,52]. For example, Blair *et al*[44] showed that median survival was significantly improved in resected PDAC patients with germline *BRCA* mutations who were treated with adjuvant PtCh compared to non-PtCh (31.0 *vs* 17.8 mo). Reiss *et al*[52] showed significant improvement in mOS in patients with unresectable PDAC and mutations in *BRCA1*, *BRCA2* or *PALB2* who were treated with PtCh compared to patients treated with non-PtCh (median follow-up of 20.1 mo *vs* mOS of 15.5 mo). Several studies have also compared the effectiveness of PtCh between patients with and without HRD mutations. In a cohort study of platinum-treated PDAC patients, patients found to have tumor-level mutations to 12 HR-related genes (including *BRCA1*, *BRCA2*, *ATM* and *PALB2*) had significantly improved median PFS compared to platinum-treated patient without HR-related gene mutations[35]. Similarly, two recent case-control studies reported improved PFS and ORR in platinum-treated patients who carried mutations to *BRCA1*, *BRCA2* and *PALB2*[53,54]. Wattenberg *et al*[53] showed an ORR of 58% in mutation carriers treated with PtCh compared to 21% non-mutated PDAC patients. In resected PDAC treated with perioperative PtCh, Yu *et al*[54] reported that mutation carriers had significantly greater survival (mOS not met *vs* 23.1 mo, HR = 0.12).

While these studies are promising, the retrospective nature introduces several limitations. Firstly, outcomes are widely subdivided as PtCh *vs* non-PtCh, however the PtCh groups generally include a variety of regimens such as gemcitabine + cisplatin, gemcitabine + oxaliplatin, FOLFOX and FOLFIRINOX. Seeing as oxaliplatin and cisplatin exert DNA damage through different mechanisms of action, it is unclear how well these findings will translate to modern clinics where patients are typically treated with FOLFIRINOX as a first-line therapy[52]. One study reported that there was no significant difference in survival for mutation-positive patients on different PtCh regimens, however in the mutation-negative group, patients only responded to FOLFIRINOX[53]. This suggests that there is potentially a role for PtCh regimens in *BRCA*-mutated patients that did not show benefit when tested in unselected PDAC populations, in situations when FOLFIRINOX cannot be tolerated. Another limitation is the current practices with respect to treatment selection. Because of the toxicity associated with PtCh such as FOLFIRINOX, these regimens are generally used in younger patients with better performance status. Therefore, in retrospective analyses of *BRCA*-mutated PDAC cohorts, it is unclear whether survival benefits seen are because of increased activity of PtCh in this patient population or because the patients treated with PtCh are younger and have better performance status. Few studies have reported data on patient age in these analyses and none have reported patient performance status. In light of this, these retrospective analyses are difficult to interpret. Lastly, retrospective studies may be affected by survival bias. Most studies compared confirmed mutation carriers to untested cohorts. It is possible that patients who survive longer are more likely to undergo genetic testing and be classified as carriers. In light of these limitations, a recent meta-analysis concluded that the current available evidence suggests PtCh is more effective in *BRCA*-mutated patients, however the quality of evidence is low[55].

To date, there have been few prospective studies assessing the effectiveness of platinum-chemotherapies in this population. A recent phase II randomized controlled trial investigated cisplatin and gemcitabine with or without Veliparib, a PARP inhibitor in patients with untreated advanced PDAC and a germline mutation of *BRCA* or *PALB2*[56]. While the primary endpoint (response rate) was not significantly different with Veliparib, the authors reported unprecedented survival rates, with a 2-year survival rate of 30.6% and a 3-year survival rate of 17.8%[56]. Response rates were also high for both arms of the study (74% with Veliparib, 65.2% without veliparib)[56]. While this data provides compelling evidence for the use of PtCh in this patient population, the study lacks a control group treated with non-PtCh for comparison. This study adds to the literature as all patients were on the same PtCh regimen (gemcitabine + cisplatin) which showed impressive responses and survival rates. Notably, the patients included in this study all had a good performance status (ECOG 0-1) and therefore these results may not translate as well to real-world PDAC patients

**Table 2 Retrospective studies of platinum-chemotherapies in *BRCA*-mutated pancreatic ductal adenocarcinoma**

| Ref. | Year | Study design | Patient population | Findings |
|---|---|---|---|---|
| Golan *et al*[43] | 2014 | Multi-institution cohort study | 71 patients with germline *BRCA* mutations (21 *BRCA1*, 49 *BRCA2*, 1 both) | Superior mOS in stage 3/4 patients treated with platinum compared to non-platinum chemotherapy (22 *vs* 9 mo, $P$ = 0.039) |
| Vyas *et al*[51] | 2015 | Cohort study | 10 patients with *BRCA2* mutation and known PDAC | Duration of response on platinum agents ranged from 8-32 wk, mean of 19.3 wk |
| Blair *et al*[44] | 2018 | Combined case control cohort study | 22 patients with resected sporadic PDAC and germline *BRCA* mutations (1 *BRCA1*, 18 *BRCA2*) | Improved OS in *BRCA*-mutated patients treated with adjuvant PtCh compared to patients treated with alternative chemotherapies or no adjuvant therapy (31.0 *vs* 17.8 *vs* 9.3 mo, $P$ < 0.001) |
| Reiss *et al*[52] | 2018 | Cohort study | 29 patients with unresectable PDAC and germline mutations of *BRCA1*, *BRCA2* or *PALB2*(12 *BRCA1*, 15 *BRCA2*, 2 *PALB2*) | Superior mOS in platinum-treated patients (undefined mOS (median follow up 21 mo) *vs* 15.5 mo, $P$ = 0.02) |
| Kondo *et al*[35] | 2018 | Cohort study | 28 patients with advanced PDAC (13 had HR-related gene mutations, 15 without mutations to HR-related genes) | Superior median PFS in HR-mutated PDAC patients treated with platinum chemotherapy compared to PDAC patients without HR mutations treated with platinum therapy (20.8 mo *vs* 1.7 mo, $P$ = 0.049) |
| Yu *et al*[54] | 2019 | Case control study | 32 resected PC patients with germline *BRCA1*, *BRCA2*, or *PALB2* mutation, 64 resected PC patient controls without germline mutations | With peri-operative platinum exposure, mOS was longer in mutation-positive group that mutation negative group (mOS not yet met *vs* 23.1 mo, HR= 0.12) |
| Wattenberg *et al*[53] | 2020 | Case control study | 26 platinum-treated patients with advanced stage PDAC and mutations of *BRCA1, BRCA2* or *PALB2*, 52 platinum-treated, wildtype, age-matched controls | Improved ORR in patients with mutations compared to controls (58% *vs* 21%, $P$ = 0.0022). Improved real world PFS in mutation carriers (10.1 mo *vs* 6.9 mo, HR = 0.43, $P$ = 0.0068) |

HR: Homologous Repair; mOS: Median overall survival; ORR: Objective response rate; PDAC: Pancreatic adenocarcinoma; PtCh: Platinum chemotherapy; *BRCA:* Breast cancer susceptibility gene.

where performance status may be lower.

Overall, there is evidence in favour of the use of PtCh as a first-line treatment for *BRCA*-mutated PDAC, however, most data is retrospective and the quality of the evidence in favour of this treatment is low. There is yet to be a randomized controlled trial confirming the observations that PtCh is preferable to other chemotherapy regimens in this population, however enrollment to such a study may be difficult due to current management practice. Furthermore, it is unclear whether or not FOLFIRINOX or gemcitabine plus cisplatin should be used for this patient population.

### *PARP inhibitors*

The sensitivity of BRCA-deficient cancers to PARP inhibition was first reported in 2005, in which researchers identified that loss of function of both *BRCA* and PARP is synthetically lethal[57,58]. PARP is an important family of enzymes involved in responding to SSB the other prominent form of DNA damage other than DSB. This combined loss of SSB repair in HRD cells is thought to lead to synthetic lethality (Figure 2). While the exact mechanism of action is still unclear, the earliest theory was that PARP inhibition prevents the repair of single-stranded DNA breaks (SSBs), leading to accumulation of replication-associated DSBs[59]. In HRD cells which have defective DSB repair, DSBs are repaired *via* error-prone NHEJ, leading to genomic instability and cell death. More recent evidence suggests that the biology of BRCA and PARP deficient synthetic lethality is more complex, however the detailed mechanisms are outside the scope of this review[60].

Therapeutic inhibitors of this pathway were evaluated in a phase I study of olaparib and confirmed activity in several different tumor types harboring *BRCA* mutations[61]. In ovarian cancer, PARP inhibitors are FDA-approved for use as a maintenance therapy in patients with recurrent ovarian cancer who demonstrated a complete or partial response to PtCh, regardless of HRD biomarker status[62]. This approval came following three phase III trials which demonstrated significant improvements in PFS in patients treated with oral PARP inhibitors as maintenance therapy following chemotherapy[63-65]. More recently, emerging data from several randomized clinical trials reporting efficacy of PARP inhibitors as a front-line treatment for newly diagnosed ovarian cancer[62]. In advanced breast cancer, PARP inhibitors have demonstrated improvements in PFS relative to chemotherapy in patients with HER2-negative, *BRCA*-mutation positive tumors[66,67]. However, there

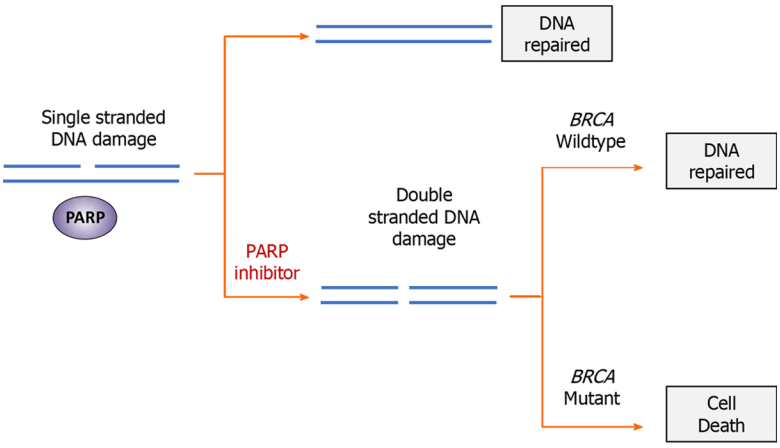


**Figure 2 Mechanism of synthetic lethality in *BRCA*-mutated cells treated with poly (ADP-ribose) polymerase inhibitors.** While neither a breast cancer susceptibility (*BRCA*) mutation or treatment with Poly (ADP-ribose) polymerase (PARP) inhibitors alone is lethal to cancer cells, dual-inhibition of both systems through mutation and pharmacological inhibition is incompatible with survival. Following PARP inhibition, single-stranded deoxyribonucleic acid (DNA) breaks are unable to be repaired. During replication, replication forks stall at unrepaired DNA damage, resulting in formation of double-stranded DNA break. In cells with defective homologous repair (*BRCA* mutations), double-stranded damage is repaired through non-homologous end joining, resulting in genomic instability and cell death. Poly (ADP-Ribose) Polymerase. PARP: Poly (ADP-ribose) polymerase; *BRCA*: Breast cancer susceptibility gene.

is yet to be a clinical trial demonstrating improvements in OS with PARP inhibitor use in advanced breast cancer[68]. Recently, PARP inhibitors have also demonstrated effectiveness in metastatic prostate cancer[69].

With the success of PARP inhibitors in other *BRCA*-associated cancers, focus has shifted to translating these findings to *BRCA*-associated PDAC. To date multiple phase II studies have evaluated the efficacy of PARP inhibitors in PDAC patients with germline *BRCA* mutations[56,70,71]. In a phase II study by Kaufman *et al*[71], 298 patients with advanced cancer (23 with pancreas cancer) and germline *BRCA1/2* mutations were treated with oral olaparib. The response rate among PC patients was 21.7% in patients who had received two prior lines of chemotherapy[71]. Conversely, another phase II study evaluated the efficacy of Veliparib in 16 advanced PDAC patients with known germline mutations of *BRCA1/2* or *PALB2* who had undergone 1-2 previous lines of treatment, finding no objective responses[70]. Authors suggested potential differences between olaparib and veliparib as a potential explanation for the difference in response rates between the two trials. Furthermore, the high rates of pre-treatment with PtCh (88% of study population) coupled with a high disease progression rate (64% of those on PtCh) may indicate a high-level of platinum-resistance in this study population, which may in turn lead to PARP inhibitor resistance[70]. This is a plausible explanation given the known association between platinum-sensitivity and PARP inhibitor sensitivity seen in ovarian cancer. Due to the tendency of cancers to develop resistance to PARP inhibitors, another approach that has been tried is combination regimens involving chemotherapy and PARP inhibitors. A recent phase II trial compared a combination regimen of gemcitabine plus cisplatin with or without veliparib as first line therapy for advanced PDAC patients with germline mutations of *BRCA1/2* or *PALB2*[56]. Veliparib did not improve response rates over gemcitabine plus cisplatin alone (74.1% *vs* 65.2%, $P$ = 0.55), however as discussed earlier, the response rates in both arms both exceeded pre-study thresholds of efficacy and therefore, the high response rate to gemcitabine plus cisplatin may have obscured any signal of benefit from veliparib.

With the relative success of combination chemotherapy regimens in PDAC (FOLFIRINOX, Gemcitabine-Abraxane), focus has been placed on the development of maintenance therapies which can prolong PFS and improve quality of life (QOL) in responders. Most recently, data from the Pancreas Cancer Olaparib Ongoing (POLO) trial has supported the use of PARP inhibitors as a maintenance therapy in this patient population following response to platinum-chemotherapy[72]. The POLO trial was an international phase III, double-blind, placebo-controlled randomized clinical trial investigating oral olaparib maintenance therapy in metastatic PDAC patients with germline *BRCA1/2* mutations who had not progressed during first-line PtCh (minimum of 16 wk of chemotherapy). Patients were randomized to either olaparib or placebo maintenance therapy. PFS was significantly longer in the olaparib group (7.4 *vs* 3.8 mo). At the time of publication, data on OS was not yet mature but preliminary

results indicated no significant difference in OS between the two groups (18.9 *vs* 18.1 mo)[72]. 18 patients (20%) in the olaparib and 6 patients (11%) in the placebo group achieved a tumor response, and the median duration of responses were 24.9 mo and 3.7 mo, respectively. Other evidence for maintenance therapy comes from the phase II study by O'Reilly *et al*[56] who reported exploratory analyses for 10 patients with germline *BRCA* or *PALB2* mutations who underwent at least 4 mo of PtCh without progression and subsequently were switched to a PARP inhibitor as maintenance therapy, finding a median PFS of 23.4 mo in this subset of patients.

In the context of maintenance therapy, preservation of quality of life and minimization of adverse effects are important goals of treatment. In the POLO trial, Grade ≥ 3 adverse events occurred in 40% of the olaparib group and 23% of the placebo group[43]. The most frequently reported adverse events in the treatment group were fatigue or anesthesia, nausea and anemia, with the majority of these cases being low grade. Only 15% and 5% of patients on olaparib underwent dose reductions or discontinued treatment because of adverse events, respectively. More recently, secondary outcomes of health-related QOL were reported, showing that olaparib treatment did not lead to a reduction in quality of life scores, a concern in the context of maintenance therapy meant to preserve functioning and QOL[73].

In light of these findings, the FDA has approved olaparib for maintenance therapy in patients with metastatic PDAC patients with germline mutations of *BRCA1/2* who have not progressed on at least 16 wk of first-line PtCh. This approval is not without controversy as there are several criticisms of the POLO trial and unanswered questions in regards to this therapy[74]. For example, the lack of improvement in OS puts the validity of the finding of improved PFS into question[74]. However, this may be because of the high rates of therapy in the placebo group following disease progression, including 15% of the patients who received a PARP inhibitor. In addition, it should be stated that the OS results were from an interim analysis with only 46% data maturity. Furthermore, concern has been raised that the discontinuation of PtCh after 16 wk in patients who were responding is incongruent with clinical practice guidelines for first-line platinum chemotherapy[74]. However, in the POLO trial, the majority of patients received FOLFIRINOX (> 80%) with a median duration of first line PtCh of 5 mo and 33% of patients receiving > 6 mo prior to randomization[72]. In addition, the PRODIGE 4/ACCORD 11 trial recommended a total of 6 mo of palliative chemotherapy[10], therefore, the duration of therapy of 1$^{st}$ line PtCh may not be out of keeping with other clinical trials in this setting of disease. Furthermore, use of placebo alone in the control group has come under criticism as evidence has emerged in favour of the continuation of 5-FU as maintenance therapy in patients who respond to FOLFIRINOX[75]. That being said, the accumulating side effects of > 4 mo of FOLFIRINOX may justify a treatment break, especially if there is no evidence of progression on imaging. Lastly, POLO only included patients with germline mutations of *BRCA1/BRCA2*, therefore it remains unclear if there is a broader population of PDAC patients who would benefit from olaparib as well, such as patients with germline mutations to other components of the HR system (*PALB2*, *ATM*) or patients with other positive biomarkers of HRD.

### *Immunotherapies*

While immunotherapies such as checkpoint inhibitors (anti-PD1/PDL1 and CTLA-4) have revolutionized the management of many cancers, they have had limited efficacy in PDAC. The genomic instability and increased total mutational load of *BRCA*-mutated and other HRD tumors results in neoantigens which may increase efficacy of immunotherapy in these tumors[11]. Recent translational studies have showed that specifically *BRCA2*-mutated tumors show increased sensitivity to immune checkpoint blockade as a result of their effect on the tumor immune microenvironment[76]. This is in line with previous findings of associations between *BRCA* mutations and PD-L1 expression in PDAC, a predictive marker for immunotherapy[77,78].

An emerging strategy for *BRCA*-mutated cancers is combination therapy with immune check point inhibitors and PARP inhibitors[79]. Given that treatment with PARP inhibitors also increases expression of PD-L1 and total mutational burden (potential biomarkers of response), combining these two therapies may act synergistically against HRD tumors[79]. In *BRCA*-mutated ovarian and breast cancers, several clinical trials are currently exploring the clinical efficacy of PARP inhibitor/immune checkpoint blockade combination therapy with early trials showing promising results[80]. In the maintenance setting, the ATHENA trial is currently testing a combination therapy consisting of rucaparib with nivolumab as a therapy for ovarian cancer following response to PtCh (NCT03522246). In PDAC, there are several ongoing Phase II trials investigating combination regimens involving PARP inhibitors

and immune checkpoint inhibitors (Table 3). The PARPVAX study is investigating combination therapy of niraparib + either ipilimumab or nivolumab as maintenance therapy following response to PtCh (NCT03404960). Another phase II study is investigating combination therapy regimens including olaparib plus durvalumab in PDAC with a primary outcomes of changes in genomic and immune markers (NCT03851614). Most recently, a study has been initiated comparing olaparib with and without pembrolizumab as maintenance therapy for *BRCA1/BRCA2* mutated-PDAC patients who responded to first-line PtCh (NCT04548752). Given the recent evidence for PARP inhibitors in PDAC, the use of immune checkpoint blockade for PDAC remains an active field of research.

## BIOMARKERS OF HRD

In the context of both PtCh and PARP inhibitors, the development of biomarkers for HRD will be an important step in implementing these therapies broadly in clinical practice. While most research to date has focused on germline mutations of *BRCA1/2* and *PALB2*, combined these represent less than 10% of all PDAC cases. While this is an important mechanism of HRD, HRD can also arise through somatic mutations or epigenetic modification of DDR genes potentially resulting in sensitivity to PtCh and PARP inhibitors. Therefore, relying solely on germline mutations of these three genes for treatment selection will likely miss patients who would otherwise benefit from targeted therapy. For example, in advanced pancreatic cancer, tumor-level mutations to HRR genes such as *BRCA1/2, ATM, PALB2, RAD51* were highly predictive of response to PtCh[35]. Recently a meta-analysis compared outcomes (ORR, survival) in PARP inhibitor trials and found that similar outcomes between patients with germline and patients with somatic *BRCA* mutations[81]. Interestingly, out of 99 studies of PARP inhibitors screened, only 18 included patients with somatic mutations, indicating that this is an understudied area of research[81]. Specifically in PDAC, only two studies investigated PARP inhibitors in patients with somatic *BRCA* mutations and both reported a non-significant increase in response rate in patients with somatic mutations, relative to germline[81]. No trials to date have evaluated the efficacy of maintenance olaparib, the only FDA-approved PARP inhibitor indication in PDAC in patients with somatic HR mutations. Two active trials of olaparib in PDAC are including patients with BRCA-associated family history or somatic HRD mutations, but explicitly excluding patients with germline *BRCA* mutations (NCT02677038, NCT02511223). However, these trials are not using olaparib in the maintenance setting. Given the efficacy of PARP inhibitors and PtCh in somatic *BRCA*-mutated ovarian cancer[63,82] this is an important area for future investigation in PDAC.

In addition to mutations of *BRCA* and other HR-related genes, genomic signatures of HRD have emerged as a promising biomarker of the HRD phenotype and subsequent treatment response[11]. These biomarkers will allow the identification sub-populations of PDAC patients who would benefit from PtCh or PARP inhibitors, and therefore expand the scope of use for these agents in PDAC. Multiple commercial assays now exist which can assess tumor tissues and assign an HRD score[62]. Examples of these assays include MyChoice CDx Assay (Myriad Genetics) and the FoundationOne CDx (Foundation Medicine) which are both FDA-approved for the evaluation of HRD. These tests combine loss-of heterozygosity scores with other markers of genomic instability (telomeric-allelic imbalance, large-scale transition) in order to quantify HRD and identify patients who would benefit from HRD-targeting therapies. These assays have been used in several clinical trials in breast and ovarian cancer and have been validated as useful biomarkers for response to PARP inhibitors[64,83,84]. Confirmation of HRD by assay is now an FDA-approved biomarker for the use of several treatment regimens including combined olaparib with bevacizumab for ovarian cancer. Furthermore, olaparib was recently approved for metastatic prostate cancer in patients with BRCA mutations or HRD. Investigating these biomarkers in PDAC will aid in identifying *BRCA*-wildtype patients who may benefit from PARP inhibitors and PtCh, an important prospect considering the poor prognosis in advanced PC.

## CONCLUSION

The field of HRD in PDAC is in its infancy relative to ovarian and breast cancers, however promising advances have been made in recent years. Currently, the available

**Table 3 Ongoing phase II clinical trials investigating poly (ADP-ribose) polymerase inhibitor/Immune Checkpoint blockade combination therapy in pancreatic ductal adenocarcinoma**

| Study identifier | Patient population | Immunotherapy | PARP inhibitor | Phase and design | Estimated completion date |
|---|---|---|---|---|---|
| NCT03404960 | Advanced PDAC patients who did not progress on PtCh | Nivolumab or Ipilimumab | Niraparib | Phase Ib/II trial evaluating effectiveness of olaparib with either nivolumab or ipilimumab | June 2021 |
| NCT03851614 | Advanced PDAC, leiomyosarcoma or mismatch repair-proficient colorectal cancer | Durvalumab | Olaparib | Phase II trial evaluating impact of combination therapy on genomic and immune biomarkers | March 2022 |
| NCT04493060 | Metastatic PDAC with mutations of *BRCA1/2* or *PALB2*, previously treated with 1-2 lines of chemotherapy including a PtCh agent | Dostarlimab | Niraparib | Phase II, evaluating the disease control rate at 12 weeks (DCR12) with combination therapy | December 2022 |
| NCT04548752 | Metastatic PDAC with germline *BRCA1* or *BRCA2* mutation treated with first-line PtCh | Pembrolizumab | Olaparib | Phase II trial comparing combination therapy to olaparib alone as maintenance therapy | March 2025 |

PDAC: Pancreatic adenocarcinoma; PtCh: Platinum chemotherapy; *BRCA*: Breast cancer susceptibility gene.

data from retrospective studies suggests that first-line PtCh is preferred however the PtCh regimen is yet to be defined. Olaparib maintenance therapy is a standard of care option in patients with *BRCA1/2* mutations and offers the benefit of ongoing anti-cancer therapy without traditional cytotoxic therapy toxicities. Important next steps include investigating these PtCh regimens and PARP inhibitors in the neoadjuvant setting, and determining if patients with somatic HR mutations or HRD as detected by genomic assays will also benefit from these treatments.

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*MINIREVIEWS*

# Metabolic and cardiovascular complications after virological cure in hepatitis C: What awaits beyond

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## Abstract

The association between chronic hepatitis C (CHC) infection and extrahepatic manifestations (EHMs), particularly cardiometabolic diseases, has been extensively examined. However, there has still been insufficient evaluation for these EHMs after virological cure. Several multidirectional mechanisms have been proposed explaining the ability of hepatitis C virus (HCV) developing EHMs, cardiometabolic ones, as well as the effect of antiviral therapy to resolve these EHMs. Data on these manifestations after achieving sustained virologic response (SVR) are still conflicting. However, current evidence suggests that reversal of hepatic steatosis and its coexistent hypocholesterolemia after successful viral eradication led to unfavorable lipid profile, which increases cardiovascular disease (CVD) risk. Additionally, most observations showed that metabolic alterations, such as insulin resistance and diabetes mellitus (DM), undergo some degree of reduction after viral clearance. These changes seem HCV-genotype dependent. Interferon-based antiviral therapy and direct acting antiviral drugs were shown to minimize incidence of DM. Large epidemiological studies that investigated the effect of SVR on CVD showed great discrepancies in terms of results, with predominant findings indicating that CVD events decreased in patients with SVR compared to non-responders or untreated ones. In this review, we present a summary of the current knowledge regarding extrahepatic sequelae of CHC following SVR, which may have an impact on healthcare providers' clinical practice.

**Key Words:** Chronic hepatitis C; Sustained virologic response; Hepatic steatosis; Diabetes mellitus; Cardiovascular disease

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**Core Tip:** The implementation of direct acting antiviral drugs has dramatically changed the landscape of hepatitis C virus (HCV) treatment, with over 95% of patients achieving sustained virologic response (SVR). Although consistent evidence demonstrated better outcomes for both hepatic and extrahepatic complications after viral clearance, data on cardiometabolic manifestation showed inconsistent results. In this review, we are shading light on the latest findings about cardiometabolic extrahepatic manifestations post-SVR. These updates may guide clinicians engaged in HCV care to integrate in their management post-viral eradication risks and subsequent long-term care.

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## INTRODUCTION

Chronic hepatitis C (CHC) infection caused by hepatitis C virus (HCV) is associated with substantial morbidity and mortality globally, affecting approximately 2.5% individuals (equivalent to 177.5 million) worldwide[1]. It is currently one of the leading etiologies for hepatocellular carcinoma (HCC) and decompensated cirrhosis requiring liver transplantation in Western countries[2,3]. The primary goal of treatment is to achieve the cure of the infection or sustained virologic response (SVR), defined as undetectable HCV RNA in the serum 12 or 24 wk after the end of treatment[4,5]. Since the introduction of first-generation direct acting antiviral agents (DAAs), boceprevir and telaprevir, in 2011, there has been a rapidly expanding population of CHC patients achieving SVR[6]. Viral eradication has been associated with marked reduction in the risk of end-stage liver disease, need for liver transplantation, and decrease in both liver-related and overall mortality[7].

Although HCV is a hepatotropic virus, for two decades several studies described the association between HCV and a heterogeneous array of extrahepatic manifestations (EHMs)[8-10] (Figure 1). Yet, the mechanism by which the virus evokes the systemic diseases remain to be elucidated. Endocrine-metabolic alterations, which are most frequently found in CHC patients, are thought to be caused by direct and indirect effects of disturbing host lipid and glucose metabolism[11,12], as well as alteration in adipocytokines released from adipose tissue[13,14]. Likewise, CHC infection has also been identified as an independent predictor for cardiovascular events such as carotid artery atherosclerosis, stroke, myocardial ischemia and heart failure, all of which are linked with poor outcomes[12]. Nevertheless, the impact on cardiovascular disease (CVD) is not fully established[15]. In clinical settings, the prognosis of CHC is not only depending on liver-related outcomes but also on extrahepatic sequelae.

Recently, much attention is drawn toward EHMs that occur following viral cure. Nonetheless, whether the development of such manifestations is a long-term consequence of the viral infection itself or an effect of HCV medications remains unknown. In this context, various reports have shown ample evidence for high prevalence of CVD and metabolic alterations such as dyslipidemia, hepatic steatosis, insulin resistance (IR), obesity and diabetes mellitus (DM)[16-19] (Table 1). While the impact of CHC infection on liver-related outcomes pre- and post-treatment has been well studied, extrahepatic sequelae, especially in post-SVR setting, are less well known. This review article highlights the current knowledge regarding the effect of SVR on EHMs.

**Table 1 Summary of studies investigated cardiometabolic manifestations of chronic hepatitis C after viral cure**

| Ref. | Antiviral regimen | The studied HCV-associated cardiometabolic manifestations | Post SVR outcomes |
|---|---|---|---|
| Fernández-Rodríguez *et al*[28], 2006 | NA[1] | Lipid disturbances. Hepatic steatosis | Hypercholesterolemia in patients with genotype 3 |
| | | | No change in genotype 3 non-responders and in patients with genotype 1 regardless of response |
| | | | Decrease in steatosis |
| Giordanino *et al*[71], 2008 | IFN monotherapy or Peg-IFN + RBV (24-48 wk) | Glucose abnormalities (IFG or DM) | No significant reduction in the risk of glucose intolerance in long-term responders and non-responders |
| Arase *et al*[70], 2009 | IFN monotherapy or IFN + RBV[2] | DM | Decreased incidence of DM in sustained responders. However, its development is associated with advanced liver disease |
| Corey *et al*[18], 2009 | NA[1] | Lipid abnormalities | Increased LDL and total cholesterol from baseline compared to non-responders |
| | | Risk of CVD | Increased CVD risk profile |
| Conjeevaram *et al*[67], 2011 | Peg-IFN + RBV (24-48 wk) | IR | Decreased IR |
| | | Obesity | Decreased in BMI |
| Kuo *et al*[27], 2011 | Peg-IFN + RBV (24 wk) | Change in serum lipid | Total cholesterol and triglycerides levels significantly increased |
| | | | No evident change in lipid profile occurred in non-SVR group |
| Aghemo *et al*[68], 2012 | Peg-IFN + RBV[2] | IR in non-diabetic CHC patients | Baseline and posttreatment HOMA-IR values were similar in SVR patients |
| | | | Significant increase in HOMA-IR was noted in non-SVR patients |
| Clark *et al*[25], 2012 | Albinterferon α-2b + RBV | Lipid abnormalities in genotypes 2,3 | Hypercholesterolemia |
| Thompson *et al*[66], 2012 | Albinterferon α-2b *vs* Peg-IFN + RBV (24-48 wk) | IR in genotypes 1,2,3 | Reduced IR in genotype 1 responders |
| | | | No change in genotype 1 non-responders and genotype 2 and 3 regardless of the response |
| Chang *et al*[29], 2014 | eg-IFN + RBV (24/48 wk) | Lipids and IR in genotypes 2, 3 | Increased total cholesterol and triglycerides in sustained responders |
| | | | Decreased HOMA-IR in patients with SVR and baseline IR |
| | | | High HOMA-IR was found in patients without baseline IR (only in genotype 1) |
| Hsu *et al*[88], 2015 | Peg-IFN + RBV (16-48 wk) | Acute coronary syndrome and ischemic stroke | Improvement in both studied circulatory outcomes |
| Innes *et al*[89], 2015 | NA[1] | CVD | Reduced hazard and absolute risk for CVD |
| Meissner *et al*[24], 2015 | SOF + RBV (24 wk) | Lipid disturbances in genotype 1 | Increased LDL level and particle size and decreased triglycerides concentration and VLDL particle size irrespective to treatment response |
| | | | Increased intrahepatic lipid-related genes in sustained responders |
| Leone *et al*[72], 2016 | IFN-based regimen | DM and CVD | No significant risk reduction in DM and CVD in SVR group as opposed to non SVR |
| Yair-Sabag *et al*[39], 2016 | Peg-IFN + RBV (24-48 wk) | IFG and DM. Triglycerides. Hepatic steatosis | Lower IFG and DM, and higher triglycerides in sustained responders |
| | | | Improvement in hepatic steatosis |
| Chang *et al*[16], 2017 | NA[1] | Cardiovascular complications | An increased adipokine PAI-1 in SVR group, which accelerates cardiovascular risk, especially in vulnerable cases |
| Mahale *et al*[69], 2018 | IFN-based regimen[2] | DM and CVD | Antiviral therapy associated with lower risk of DM and stroke whereas no significant effect on CVD |

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|---|---|---|---|
| Nahon *et al*[90], 2017 | Peg-IFN + RBV (16-48 wk) or combination therapies[3] | CVD | Lower risk of CVD in SVR subjects in comparison to non SVR |
| Stine *et al*[74], 2017 | DAAs[2,3] | DM in genotypes 1, 2, 3 | Glycosylated hemoglobin was not affected in known diabetic patients<br>1/3 of patients required escalation of anti-diabetic therapy during antiviral treatment |
| Carvalho *et al*[11], 2018 | SOF + LDV ± RBV (group 1) *vs* Peg-IFN + RBV (group 2) | Lipid levels. Serum glucose. IR | While total cholesterol increased in both groups, triglycerides levels decreased in group 1 and increased in group 2<br>LDL elevated in group 1 and No change in group 2<br>No significant variation in serum glucose<br>Significant increase in HOMA-IR only in group 2 |
| Kawagishi *et al*[17], 2018 | DAAs[3] | Hepatic steatosis. Lipid abnormalities | Decrease in CAP and LDL in patients with high baseline values<br>Elevated sdLDL in patients who had dyslipidemia and hepatic steatosis at 24 wk |
| Li *et al*[73], 2018 | DAAs[4] | DM | Lower risk of DM in SVR patients than in treatment failure group |
| Noureddin *et al*[46], 2018 | DAAs[3] | Hepatic steatosis and fibrosis | High prevalence of fatty liver<br>Although fibrosis has been reduced in patients with and without steatosis compared to baseline, patients with steatosis continued to have clinically significant liver stiffness |
| Li *et al*[10], 2019 | IFN + RBV (48 wk) | Serum glucose level and IR | Reduced glucose level<br>Improved IR |
| Butt *et al*[87], 2019 | IFN + RBV[2,3].DAAs[2,3] | CVD | Lower incidence in treatment group, compared to controls<br>DAAs showed greater risk reduction than interferon-based regimen<br>SVR associated with decreased CVD risk |
| Abdo *et al*[75], 2020 | SOF + DCV (12-24 wk) | Glycemic status, IR, and lipid profile in CHC patients with DM | Improvement of glycemic state and HOMA-IR<br>Global worsening of lipid profile |
| Graf *et al*[45], 2020 | DAAs[3] | IR, lipid perturbations, body weight changes, and hepatic steatosis | Lower HOMA-IR compared to baseline<br>Higher total cholesterol, LDL, and HDL<br>Higher CAP relative to baseline<br>BMI did not significantly change over time |
| Huang *et al*[31], 2020 | DAAs[4] | Lipids and cardiovascular events | Increased total cholesterol and LDL<br>Higher cardio-cerebral diseases |

[1]No data available on antiviral therapy.
[2]Data on course of treatment is not available.
[3]Various regimens were used.
[4]No data available neither on types of direct acting antiviral agents nor on treatment duration. BMI: Body mass index; CAP: Controlled attenuation parameter; CHC: Chronic hepatitis C; CVD: Cardiovascular disease; DAAs: Direct acting antiviral agents; DCV: Daclatasvir; DM: Diabetes mellitus; HDL: High density lipoprotein cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; IFG: Impaired fasting glucose; IFN: Interferon; IR: Insulin resistance; LDL: Low-density lipoprotein cholesterol; LDV: Ledipasvir; NA: Not available; PAI-1: Plasminogen activator inhibitor-1; Peg-IFN: Pegylated interferon; RBV: Ribavirin; sdLDL: Small-dense low-density lipoprotein; SOF: Sofosbuvir; SVR: Sustained virologic response; VLDL: Very low-density lipoprotein cholesterol; HCV: Hepatitis C virus.

## DYSLIPIDEMIA

It has been known that HCV possesses a mutual relationship with host lipids and lipoproteins metabolisms, which the virus uses for multiple key steps in its life cycle[20,21]. HCV circulates as a lipid-rich particle, utilizing lipoprotein cell receptors to gain entry into the hepatocyte[22,23]. Within hepatocytes, it influences three mechanisms in lipid metabolism: It upregulates lipid biosynthesis, impairs

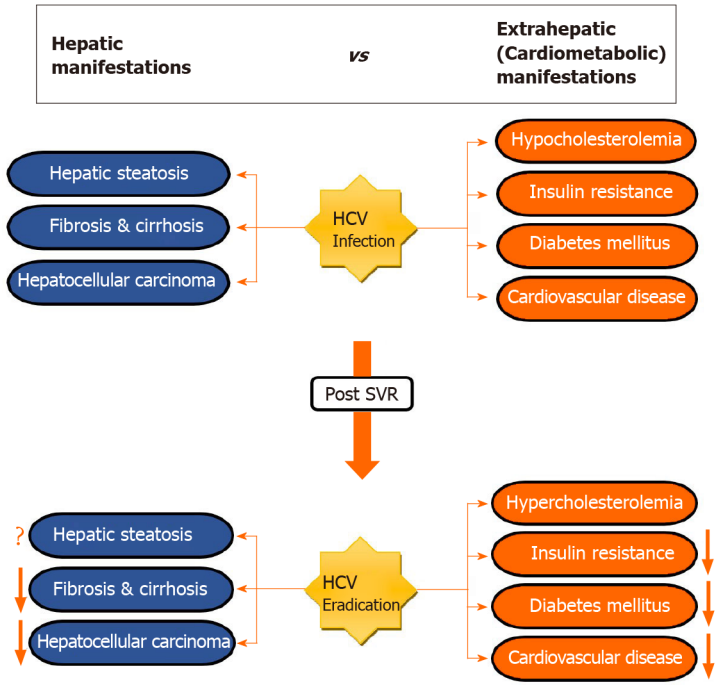


**Figure 1 Hepatic and extrahepatic (cardiometabolic) manifestations associated with hepatitis C virus infection and the effect of sustained virologic response on the cardiometabolic manifestations based on most of the evidence.** HCV: Hepatitis C virus; SVR: Sustained virologic response.

mitochondrial β-oxidation and thus lipid degradation, and reduces apolipoprotein exportation, in particular very low-density lipoprotein cholesterol (LDL), resulting in significant intracellular lipid accumulation and circulating hypocholesterolemia and hypolipoproteinemia[13].

Several studies have linked successful HCV eradication with rebound rise in lipid levels. Meissner *et al*[24], who investigated the influence of DAAs, sofosbuvir and ribavirin, on serum lipid profiles and intrahepatic lipid-related genes expression in patients with genotype 1 CHC, reported that serum LDL level and molecular size increased early in therapy, whereas triglycerides concentration and very low-density lipoprotein cholesterol (VLDL) particle size decreased concomitantly, irrespective of treatment outcome. This observation likely reflects a direct effect on lipid metabolism associated with the inhibition of HCV replication[24]. This notion was further supported in several reports. Clark *et al*[25], used cholesterol metabolites as an indicator to evaluate the impact of HCV on lipid metabolism. In this study, genotype 3 but not genotype 2 showed a selected interference with late cholesterol synthesis pathway, resulting in hypocholesterolemia. However, this interference was resolved after SVR. Another Japanese study that included 100 subjects showed early rebound (within 28 d) in LDL level in CHC patients who underwent interferon-free DAA treatment. However, the elevation was regimen-specific, more prominent in the group who received daclatasvir and asunaprevir for 24 wk than in those received ledipasvir and sofosbuvir for 12 wk[26]. In addition, many reports correlated the rebound in lipid profile with treatment response status[27-29]. This was clearly demonstrated by Corey *et al*[18], which conducted a 2 steps study to evaluate the relationship between CHC infection and its treatment with lipid levels. After confirming that HCV infection is associated with significantly lower LDL concentrations in the first step, they found that remarkable hyperlipidemia was developed in patients who achieved viral clearance, compared to non-responders or those who relapsed. In the same context, some studies have further investigated the role HCV genotype on post-SVR hypercholesterolemia. In a study that included 215 patients, Fernández-Rodríguez *et al*[28] observed that increased serum cholesterol levels were associated with genotype 3 in patients who achieved SVR. In contrast, un-changed serum cholesterol figures were noted in genotype 3 non-responders and genotype 1 regardless of response[28]. Although the reversal of both hepatic steatosis and hypolipidemia has been reported only in genotype 3 in this study, there is accumulating evidence demonstrating that the reversal of hypolipidemia is not HCV-genotype specific[29,30].

Many reports proposed that atherosclerotic CVD risk increases after successful eradication of HCV due to the unfavorable lipid profile, which is a result of reversed hypolipidemia, represented in high serum LDL and small dense LDL. The latter has greater atherogenic potential and is a better marker for prediction of CVD than LDL[31,32]. The important question at this point is whether these patients require lipid-lowering treatment. According to the National Cholesterol Education Program Adult Treatment Plan Guideline III, patients should be put on lipid lowering agents for: (1) an LDL >100 mg/dL, if they have coronary heart disease or its equivalents[1]; (2) an LDL >130 mg/dL, if they have two or more major coronary heart disease risk factors[2]; and (3) an LDL >190 mg/dL, with none or one major risk factor[33]. Corey and his colleagues have found that 13% of their studied cohort had post-SVR LDL levels requiring lipid lowering therapy as these patients had values > 130 mg/dL plus presence of two or more major coronary heart disease risk factors. Nonetheless, before antiviral therapy, none of these patients had LDL readings requiring medications. Post-treatment lipid profile deterioration may reach clinically meaningful level requiring the consideration for cholesterol lowering therapy.

## HEPATIC STEATOSIS

Hepatic steatosis is a frequent histological liver finding in patients with CHC[34]. Since HCV is known to hijack lipid metabolic pathways for virion maturation and secretion, several possible mechanisms of HCV-induced liver steatosis have been suggested. HCV induces lipogenesis by increasing intrahepatic fat milieu through sterol regulatory element binding protein 1c, which is a protein that overexpresses LDL receptors which in turn facilitates fatty acid uptake by hepatocytes, leading to higher intrahepatic fat content[35]. In contrast, HCV inhibits lipolysis by disturbing mitochondrial β-oxidation[36], either directly by the virus itself or indirectly *via* downregulation of the enzyme carnitine palmitoyltransferase-1, which regulates fatty acids oxidation[37,38]. These two mechanisms further potentiated by HCV-induced IR[39]. Moreover, HCV core protein suppresses the activity of microsomal triacylglycerol transfer protein, which is used for the assembly and secretion of VLDL, resulting in increased intracytoplasmic lipid droplets and therefore steatosis[40]. Miyoshi *et al*[41], who studied the role of HCV core protein in development of steatosis in HCV genotype 2, revealed that core protein activates the enzyme δ-9 desaturase, fatty acid metabolizing enzyme, and therefore leads to accumulation of triglycerides. This lipid metabolism disorder was also associated with mitochondrial dysfunction[41].

Hepatic steatosis is commonly reported among patients with HCV genotype 1 and genotype 3. Its occurrence in the latter has been correlated mainly to the previously mentioned mechanisms. Therefore, resolution of steatosis observed after successful viral eradication suggests a direct steatogenic pathway for HCV genotype 3[42]. This hypothesis was backed in a study of patients treated with interferon-based regimen, in which 91% of genotype 3 patients and only 43% of other genotypes have had their steatosis improved after viral cure[43]. Kumar *et al*[44], have also observed similar findings when steatosis was profoundly reduced in genotype 3 patients post-SVR, while no change irrespective of the treatment response occurred in genotype 1. Although development of fatty liver was associated with viral characteristics in genotype 3 (viral steatosis), the condition in genotype 1 corresponded to metabolic features such as glucose level and IR (metabolic steatosis). This observation suggests that in patients with genotype 1, factors other than the viral features play an essential role in the development of hepatic steatosis[28].

After achieving SVR with antiviral therapy, reversal of steatosis is the most common reported outcome, which was seen in several studies[17,27,28,39]. However, recent reviews showed contradictory findings[45,46]. In a prospective study that investigated the prevalence of hepatic steatosis and fibrosis in patients with CHC post-SVR, steatosis prevalence found to be 47.5%, almost as same as the pre-treatment figure (50%). Besides, overall average fibrosis score was reduced after viral clearance. Nevertheless, patients who had steatosis have maintained clinically significant fibrosis scores, compared to those without fatty liver[46]. In another study included 49 patients aimed to evaluate the impact of DAAs on glucose and lipid homeostasis, controlled attenuation parameter values were markedly increased at the end of follow up compared to baseline. More importantly, this finding was independent of weight gain, since no change in body mass index (BMI) was observed over time[45].

Patients with CHC and viral-induced hepatic steatosis have been shown to have worse hepatic outcomes in pre-treatment setting[47]. Nonetheless, a recent study depicts that presence of post-SVR steatosis does not carry a better risk profile[48]. In this study, which aimed to assess the effect of steatosis on HCC and all-cause mortality in CHC patients post-SVR, presence of fatty liver was associated with a considerable 7.5-fold increase in both primary endpoints[48]. Furthermore, there is also a substantially higher risk of EHMs, particularly CVD, after amelioration of steatosis post-SVR[17]. These findings combined highlight the importance of hepatic steatosis as a major risk factor for poor outcome and warrant a special consideration of screening and follow-up in this population.

## INSULIN RESISTANCE & DIABETES MELLITUS

Based on multiple epidemiological studies, metabolic alterations such as IR, DM, and metabolic syndrome are frequent comorbidities in patients with CHC, as opposed to controls[19,49]. The rationale behind this association is still not completely understood but it could be attributed to the presence of liver disease, metabolic characteristics such as obesity, or the inflammatory process induced by HCV infection. HCV has been found to modulate insulin signaling pathways although the precise molecular mechanism of HCV-mediated IR is not fully understood. In two mouse-model experimental studies, HCV core protein was found to play a major role in the development of IR[50,51] particularly through *PA28γ* gene-dependent pathway[50]. HCV genotypes 1, 2 and 4[52] and genotypes 1 and 4[53] were noticed to have higher IR compared to genotype 3[52] and genotypes 2 and 3[53], respectively. Oxidative stress and proinflammatory cytokines were also found to play a role in *de novo* IR[54,55]. The disruption in glucose and lipid metabolism associated with IR[56] leads to evolution of hepatic steatosis and development of DM. Among subjects with chronic liver disease, the prevalence of DM in CHC patients prior to treatment varies from 13.6% to 67.4%, which is higher than that reported in individuals with other etiologies, such as chronic hepatitis B[57]. Furthermore, a case-control study demonstrated that the presence of CHC was associated with an over 11-fold increase in risk of developing DM over a follow-up period of 9 years[58]. DM seems to have a bidirectional relationship with HCV, in which the latter causes IR while DM is linked with more aggressive course of HCV-related outcomes such as progressive fibrosis[49,59,60], and increased risk of cirrhosis and HCC[61,62]. All the above conditions make patients with CHC more susceptible to have metabolic syndrome[63]. However, due to the hypolipidemia caused by HCV infection[64], which does not fit the traditional diagnostic criteria, a peculiar type of metabolic syndrome known as hepatitis C-associated dysmetabolic syndrome has been defined[63,65].

There is frequent evidence that have showed a beneficial effect of antiviral therapy using interferon-based regimens on IR in long-term HCV responders. Thompson *et al*[66], who studied 1038 non-diabetic patients, concluded that IR was substantially decreased in HCV-genotype 1 responders but not in genotype 1 non-responders or those with genotype 2 or 3 irrespective of treatment outcome. This finding was independent of any changes in BMI. Similar findings were also reported in a prospective study[29]. In the Virahep-C, a prospective multicenter study, an improvement in the homeostatic model assessment for IR (HOMA-IR) was observed 24 wk after treatment completion among HCV genotype 1 patients who had IR prior to therapy[67]. Nonetheless, Aghemo *et al*[68], who enrolled 384 non-diabetic patients with HCV genotypes 1 and 4 failed to display any differences in HOMA-IR values between baseline and 24 wk post-SVR. All the above findings indicate that longer follow-up may be needed to better assess glucose metabolism disturbances after HCV viral clearance with interferon-based regimens, especially in HCV genotype 1 patients. Paradoxically, in a head-to-head comparison of 178 subjects with HCV genotype 1 and 4 between interferon-based antiviral therapy and DAAs to assess metabolic outcomes, there was a significant elevation in HOMA-IR in those who have taken interferon-based regimen[11].

In addition to its effect on IR, antiviral therapy has been thought to decrease incidence of post-SVR hyperglycemia and DM. Interferon-based regimens have been studied extensively and they are usually associated with a decreased incidence of DM in non-diabetic patients with CHC after elimination of HCV[69]. However, several studies have emphasized the beneficial role of attaining SVR, which lessens glucose metabolism abnormalities induced by HCV infection[10,39,70]. Other studies could not detect any significant differences between treatment responders and non-

responders[71,72]. Despite these conflicting results, a meta-analysis that included seven studies aiming to investigate the correlation between HCV clearance *via* interferon-based regimens and the incidence of hyperglycemia demonstrated that SVR is associated with lower risk of hyperglycemia [odds ratio 0.49, 95% confidence interval (CI): 0.42-0.58]. Heterogeneity between studies was minimal, indicating a reliable result. On the other hand, the use of DAAs for viral eradication was not investigated thoroughly. Studies on incidence of DM in non-diabetic patients demonstrated less glucose disturbance in long-term responders[10,73]. In a retrospective study conducted in United States, 5127 non-diabetic subjects with HCV were enrolled to investigate how the response to HCV treatment impacted the risk of subsequent DM. The authors found that those who achieved SVR had markedly lower risk of developing DM, compared to those with treatment failure[10]. Two studies investigated the effect of DAAs on previously known diabetic patients[74,75]. One illustrated an improvement in glycemic status after viral cure while in the other there was no difference in glycosylated hemoglobin between pretreatment and post-treatment values. Importantly, one-third of patients in the latter study required escalation of anti-diabetic therapy during antiviral treatment. Further long-term prospective studies are still needed to resolve the current dilemma of changes on IR related to antiviral treatment.

## CARDIOVASCULAR DISEASE

CHC infection has been linked to an array of EHMs, including an increased risk of CVD[76-79]. Several direct and indirect HCV pro-atherogenic mechanisms have been postulated. HCV is assumed to play a direct role in the development of arterial atherosclerosis by inducing endothelial dysfunction, likely through interleukin 1β[80], a pro-inflammatory cytokine. Likewise, it has been observed that HCV has the ability to live and replicate inside carotid plaques[81], which further supports an immediate pro-atherogenic effect. Moreover, chronic inflammation and oxidative stress that are caused by structural and non-structural viral proteins have also been shown to trigger plaque formation[80]. In a multicenter Italian study that evaluated the effect of attaining SVR using DAAs on subclinical carotid arteriosclerosis compared to an untreated cohort, ultrasonographic carotid measurements showed a significant reduction in mean carotid intima-media thickness in treatment group at the end of follow-up compared to baseline (from 0.94 mm to 0.81 mm, $P < 0.001$). No significant changes in the intima-media thickness were found in the control group. The BMI of these patients did not change during follow-up, while a significant increase in serum cholesterol levels was observed. The study concluded that eradication of HCV by DAAs led to an amelioration in carotid atherosclerosis, particularly intima-media thickness. Furthermore, HCV can also induce atherosclerosis indirectly since it is associated with an increased risk of metabolic syndrome components, including IR, DM, and hepatic steatosis, which are well-known predisposing factors for CVD[82-84]. On the other hand, some studies have failed to show any significant association between HCV and cardiovascular events[85,86].

Several studies have shown that either antiviral therapy or the attainment of SVR minimize CVD risk[87-90]. However, the results are rather controversial. Butt *et al*[87], who studied the effect of antiviral therapy, interferon- or DAAs-based regimens, on CVD risk found that the incidence of CVD in treatment arm was 7.2% in comparison with 13% in control group, regardless of the antiviral regimen. Treatment with DAAs was superior to interferon-based regimen, with a hazard ratio (HR) of 0.57 (95%CI: 0.51-0.65) and HR 0.78 (95%CI: 0.71-0.85), respectively. SVR was also associated with lower risk of incident CVD events HR 0.87 (95%CI: 0.77-0.98). In a nation-wide cohort study on Taiwanese residents with HCV who had received interferon-based regimens compared to an untreated cohort, antiviral therapy was associated with lower risks of acute coronary syndrome and ischemic stroke, with HR 0.77 (95%CI: 0.62-0.97) and HR 0.62 (95%CI: 0.46-0.83), respectively. This risk reduction was not observed in subject who had insufficient treatment course (< 16 wk)[88]. Further supporting data was observed in a study comprising 3385 HCV patients, which found that SVR was associated with a lower relative hazard reduction and absolute risk reduction for CVD[89]. However, some epidemiological studies have found contradictory findings. A large retrospective cohort study which enrolled 160875 subjects was aimed to investigate the impact of successful viral eradication on a variety of EHMs. In terms of CVD risk, the study concluded that SVR was associated with a diminished risk for stroke HR 0.84 (95%CI: 0.74-0.94), but not for CVD aHR 1.12 (95%CI: 0.81-1.56), when

compared to the untreated cohort[69]. From the same perspective, a negative result was also reported by Leone *et al*[72], who studied the influence of SVR on EHMs. The researchers did not find any significant cardiovascular risk reduction in SVR group compared to non-SVR, with HR 1.14 (95%CI: 0.57-2.3). Despite disparities in the findings across individual studies, a meta-analysis including 53841 patients demonstrated that SVR significantly reduces CVD risk, with a pooled of HR 0.76 (95%CI: 0.61-0.94)[91].

Apart from the direct treatment effect on CVD risk, therapeutic changes on other EHMs may also play role in the development of atherosclerotic events. Deteriorated lipid profile after HCV clearance has been shown to predispose patients to an elevated risk of CVD[31]. In a study of 617 patients with a mean follow-up of 26.8 mo, Huang *et al*[31] investigated whether deterioration of lipid profile post-SVR increased the risk of cardio-cerebral disease. Five patients developed cardio-cerebrovascular events (3 CVD and 2 cerebrovascular disease) over 1376 person-years. An LDL surge >40% was found to be the only predictor of these vascular events, with a HR of 15.44 (95%CI: 1.73-138.20)[31]. Evidence on risk of CVD in CHC pre- or post-treatment remains contro-versial. Nonetheless, most of the literature indicates that achieving SVR *via* antiviral therapy is associated with a significant risk reduction.

## CONCLUSION

EHMs including cardiometabolic conditions are commonly seen among patients with CHC infection. Data on these conditions after elimination of HCV is inconsistent. However, the predominant evidence in the literature suggests that viral clearance using antiviral therapy leads to deterioration in lipid profile, reduction in the incidence of metabolic alteration such as IR, DM, and hepatic steatosis, and improvement in CVD risk. To determine more robust level of association between SVR and EHMs and to understand the exact mechanisms of how antiviral therapies act on these EHMs, large prospective studies with long-term follow-up are needed.

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ORIGINAL ARTICLE

Basic Study

# S100 calcium binding protein A6 and associated long noncoding ribonucleic acids as biomarkers in the diagnosis and staging of primary biliary cholangitis

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## Abstract

### *BACKGROUND*

Primary biliary cholangitis (PBC) is a chronic and slowly progressing cholestatic disease, which causes damage to the small intrahepatic bile duct by immuno-regulation, and may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure.

### *AIM*

To explore the potential diagnosis and staging value of plasma S100 calcium binding protein A6 (S100A6) messenger ribonucleic acid (mRNA), LINC00312, LINC00472, and LINC01257 in primary biliary cholangitis.

### *METHODS*

A total of 145 PBC patients and 110 healthy controls (HCs) were enrolled. Among them, 80 PBC patients and 60 HCs were used as the training set, and 65 PBC patients and 50 HCs were used as the validation set. The relative expression levels of plasma S100A6 mRNA, long noncoding ribonucleic acids LINC00312, LINC00472 and LINC01257 were analyzed using quantitative reverse transcription-polymerase chain reaction. The bile duct ligation (BDL) mouse model was used to simulate PBC. Then double immunofluorescence was conducted to verify the overexpression of S100A6 protein in intrahepatic bile duct cells of BDL mice. Human intrahepatic biliary epithelial cells were treated with glycochenodeoxycholate to simulate the cholestatic environment of intrahepatic biliary epithelial cells in PBC.

### *RESULTS*

The expression of S100A6 protein in intrahepatic bile duct cells was up-regulated in the BDL mouse model compared with sham mice. The relative expression

internationally accepted principles for the care and use of laboratory animals [China Medical University Application for Laboratory Animal Welfare and Ethical review (201702 Edition)].

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levels of plasma S100A6 mRNA, log10 LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in plasma of PBC patients compared with HCs (3.01 ± 1.04 *vs* 2.09 ± 0.87, $P < 0.0001$; 2.46 ± 1.03 *vs* 1.77 ± 0.84, $P < 0.0001$; 3.49 ± 1.64 *vs* 2.37 ± 0.96, $P < 0.0001$; 1.70 ± 0.33 *vs* 2.07 ± 0.53, $P < 0.0001$, respectively). The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate compared with control (2.97 ± 0.43 *vs* 1.09 ± 0.08, $P = 0.0018$; 2.70 ± 0.26 *vs* 1.10 ± 0.10, $P = 0.0006$; 2.23 ± 0.21 *vs* 1.10 ± 0.10, $P = 0.0011$; 1.20 ± 0.04 *vs* 3.03 ± 0.15, $P < 0.0001$, respectively). The mean expression of S100A6 in the advanced stage (III and IV) of PBC was up-regulated compared to that in HCs and the early stage (II) (3.38 ± 0.71 *vs* 2.09 ± 0.87, $P < 0.0001$; 3.38 ± 0.71 *vs* 2.57 ± 1.21, $P = 0.0003$, respectively); and in the early stage (II), it was higher than that in HCs (2.57 ± 1.21 *vs* 2.09 ± 0.87, $P = 0.03$). The mean expression of LINC00312 in the advanced stage was lower than that in the early stage and HCs (1.39 ± 0.29 *vs* 1.56 ± 0.33, $P = 0.01$; 1.39 ± 0.29 *vs* 2.07 ± 0.53, $P < 0.0001$, respectively); in addition, the mean expression of LINC00312 in the early stage was lower than that in HCs (1.56 ± 0.33 *vs* 2.07 ± 0.53, $P < 0.0001$). The mean expression of log10 LINC00472 in the advanced stage was higher than those in the early stage and HCs (2.99 ± 0.87 *vs* 1.81 ± 0.83, $P < 0.0001$; 2.99 ± 0.87 *vs* 1.77 ± 0.84, $P < 0.0001$, respectively). The mean expression of LINC01257 in both the early stage and advanced stage were up-regulated compared with HCs (3.88 ± 1.55 *vs* 2.37 ± 0.96, $P < 0.0001$; 3.57 ± 1.79 *vs* 2.37 ± 0.96, $P < 0.0001$, respectively). The areas under the curves (AUC) for S100A6, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.759, 0.7292, 0.6942 and 0.7158, respectively. Furthermore, the AUC for these four genes in PBC staging were 0.666, 0.661, 0.839 and 0.5549, respectively. The expression levels of S100A6 mRNA, log10 LINC00472, and LINC01257 in plasma of PBC patients were decreased (2.35 ± 1.02 *vs* 3.06 ± 1.04, $P = 0.0018$; 1.99 ± 0.83 *vs* 2.33 ± 0.96, $P = 0.036$; 2.84 ± 0.92 *vs* 3.69 ± 1.54, $P = 0.0006$), and the expression level of LINC00312 was increased (1.95 ± 0.35 *vs* 1.73 ± 0.32, $P = 0.0007$) after treatment compared with before treatment using the paired *t*-test. Relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r = 0.683$, $P < 0.0001$); serum level of collagen type IV was positively correlated with the relative expression of log10 LINC00472 ($r = 0.482$, $P < 0.0001$); relative expression of S100A6 mRNA was positively correlated with the serum level of collagen type IV ($r = 0.732$, $P < 0.0001$). The AUC for the four biomarkers obtained in the validation set were close to the training set.

## *CONCLUSION*

These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a potential biomarker for staging in PBC.

**Key Words:** S100 calcium binding protein A6; Long noncoding ribonucleic acids; Primary biliary cholangitis; Biomarker; Diagnosis; Staging



**Core Tip:** Primary biliary cholangitis (PBC) is an autoimmune liver disease which is characterized by intrahepatic cholestasis. The expression of S100 calcium binding protein A6 (S100A6) was up-regulated in a bile duct ligation mouse model compared with sham mice. The relative expression levels of plasma S100A6 messenger ribonucleic acid, LINC00472 and LINC01257 were up-regulated and the relative expression of LINC00312 was down-regulated in PBC patients. S100A6 and the three long noncoding ribonucleic acids can be used as biomarkers for PBC diagnosis and staging using receiver operating characteristic curve analysis. The results were further verified *in vitro* using intrahepatic biliary epithelial cells.

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## INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic and slowly progressing cholestatic disease, which causes damage to the small intrahepatic bile duct by immuno-regulation, and may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells (iBECs) is the key to investigate the pathogenesis of PBC, but the accurate relationship between cholestasis and liver fibrosis is still indistinct. Currently, liver injury caused by cholestasis is mainly studied using liver cell lines or liver cancer cell lines treated with hydrophobic bile acids[1], while iBECs, the main target cells of PBC, have rarely been studied.

S100 calcium binding protein A6 (S100A6), also known as calcyclin, is a $Ca^{2+}$ binding protein and is a member of the S100 family. Its distribution in the body is specific to cells and tissues, having a high expression in normal epithelial cells and fibroblasts, as well as in some tumor cells[2]. As an intracellular protein, S100A6 is involved in the regulation of various cellular functions, such as proliferation, apoptosis, cytoskeletal dynamics, and cell response to different stressors. It is believed that S100A6 may be involved in the ubiquitination of beta catenin and play an important role in controlling the cell cycle process[3]. S100A6 can interact with the calcyclin-binding protein/Siah-1-interacting protein, which is a component of the ubiquitin ligase complex[4].

Long non-coding ribonucleic acids (lncRNAs) are involved in the regulation of a variety of intracellular processes[5]. As a structural component, lncRNAs can form a nucleic acid protein complex with gene regulatory transcription factors[6]. LncRNAs can also bind to specific transcription factors and change their cellular localization, thus affecting the transcription of target genes. Abnormal expression of lncRNAs in plasma has been shown to accurately predict several human diseases[7,8].

As a general rule, PBC diagnosis depends on titers of antimitochondrial antibody (AMA), serum level of alkaline phosphatase (ALP) and liver biopsy[9-11]. However, it is difficult to achieve an early diagnosis in AMA-negative patients, or to differentiate from other autoimmune liver diseases; thus, an invasive liver biopsy is required to make a definitive diagnosis, and this not only increases the financial burden of patients, but also brings mental and physical trauma to patients, often delaying the best time for treatment. However, after definite diagnosis, some patients fail to respond to ursodeoxycholic acid treatment and often have a poor prognosis or even progress to liver failure. The majority of PBC cases are diagnosed mostly at an advanced stage, so diagnosis and staging biomarkers of PBC are urgently needed.

In this study, we explored the value of S100A6 and its associated lncRNAs as potential biomarkers for the diagnosis and staging of PBC.

## MATERIALS AND METHODS

### *Study design*

This study included three phases (Figure 1): (1) The discovery phase, in which candidate genes and lncRNAs were searched using bioinformatics methods, and were then verified by a mouse model and cell model; (2) The training phase, in which quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to evaluate the relative expression levels of target gene and lncRNAs in the plasma of PBC patients and healthy controls, as well as to estimate their diagnosis and staging value; and (3) the validation phase, in which the diagnosis and staging value of target genes and lncRNAs was verified in another independent PBC cohort.

### *Identification of differentially expressed genes from the gene expression omnibus dataset*

The GSE29776 array dataset was analyzed on the gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/)[12]. The dataset contains 6 mouse liver tissue samples, including 3 bile duct ligation (BDL) mouse samples and 3 sham mouse samples. "GEO2R" in the webpage was used to analyze the array database.

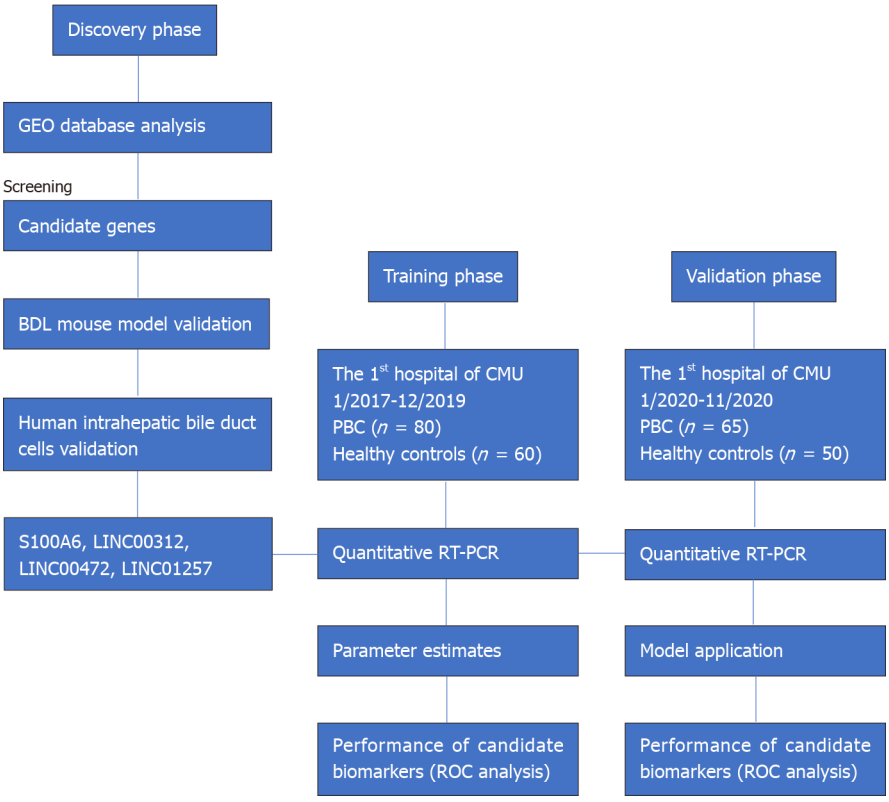


**Figure 1 Study design.** BDL: Bile duct ligation; GEO: Gene Expression Omnibus; PBC: Primary biliary cholangitis; ROC: Receiver operating characteristic curve; RT-PCR: Reverse transcriptase polymerase chain reaction.

### *LncRNAs selection*

The PROMO usage database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to predict the transcription factors of the S100A6 promoter[13]. There were multiple binding sites between the transcription factor estrogen receptor alpha (also known as ESR1) and the promoter of S100A6. Experiments with BDL mice and PBC patients suggested that the expression of estrogen receptor in bile duct epithelial cells was associated with cholestasis or bile duct epithelial cells in PBC[14]. We hypothesized that ESR1 could regulate the transcription of S100A6 as a transcription factor and thus play an important role in the injury of bile duct cells in PBC. The Gene-Cloud of Biotechnology Information database (https://www.gcbi.com.cn/gcanalyze/html/generadar/index) was used to screen lncRNAs associated with ESR1[15]. The binding force between lncRNAs and ESR1 was calculated by RNA-Protein Interaction Prediction (http://pridb.gdcb.iastate.edu/RPISeq/)[16]. As RF and SVM scores of LINC00312, LINC00472, and LINC01257 were all found to be close to 1.0, these three lncRNAs were selected as candidate lncRNAs in this study.

### *Animal studies, bile duct ligation model*

Male C57BL/6J mice (aged 6-8 wk) were purchased from the Animal Experiment Department of China Medical University (Shenyang, Liaoning Province, China). All mice were weighed and randomly grouped with an average weight of 20-25 g into the BDL group and the sham group. To simulate cholestasis, 9 mice underwent BDL[17]. The animal protocol was designed to minimize pain or discomfort to the mice. The animals were acclimatized to laboratory conditions (24 °C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. The BDL procedure was performed with the common bile duct doubly ligated under anesthesia *via* laparotomy[18]. The sham procedure was performed *via* a similar laparotomy without BDL. Animal experiments were approved by the Ethics Committee of the Animal Experiment Department of China Medical University.

A portion of the liver tissue was placed in a 4% p-formaldehyde solution and routinely processed for histological assessment, while the remaining tissue was snap frozen and stored at -80 °C.

### Histological analysis

The mice were sacrificed by cervical dislocation and the liver was immediately removed by laparotomy. Part of the right lobe of the liver was fixed in 4% formaldehyde. The liver tissues were embedded in paraffin and sliced. Hematoxylin and eosin stained liver sections were observed under a light microscope at x 400 magnification to evaluate whether the cholestasis model was successfully established[19].

### Double immunofluorescence

To identify whether the expression of S100A6 protein was up-regulated in bile duct epithelial cells in BDL mice, we performed double immunofluorescence[20] for S100A6 antibodies (Abcam, USA, Cat. No. ab181975) with cytokeratin 19 (CK19) antibodies (Abcam, USA, Cat. No. ab52625) which was specifically expressed in epithelial cells[21]. The primary antibody was replaced by rabbit or mouse IgG for negative controls. The working concentration of fluorescein isothiocyanate and tetraethyl rhodamine isothiocyanate was 1:50. Nuclei were counterstained with DAPI. The empirical procedure was performed according to the manufacturer's instructions. The sections were counterstained with DAPI and evaluated under a conventional fluorescence microscope.

### Cells culture and treatments

Human intrahepatic biliary epithelial cells (HiBECs) were purchased from Guangzhou Jennio Biotech Company Limited (Guangzhou, Guangdong Province, China). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (GEMINI, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in incubators at 37 °C with 5% $CO_2$. HiBECs were treated with 1000 mmol/L glycochenodeoxycholate (GCDC)[22] for 24 h to mimic cholestasis in PBC patients.

### Patients

A total of 80 untreated PBC patients and 60 healthy controls as the training set were enrolled in order to differentially evaluate S100A6 and lncRNAs. In addition, another cohort consisting of 65 PBC patients and 50 healthy controls was used as the validation set. PBC patients were diagnosed by the Department of Gastroenterology or Rheumatology of The First Affiliated Hospital of China Medical University between January 2017 and November 2020. The diagnosis of PBC needed to meet two of the following three criteria[9-11]: (1) AMA titer > 1:40; (2) ALP level 1.5-times higher than the normal upper limit for more than 24 wk; and (3) liver biopsy revealing non-suppurative cholangitis and interlobular bile duct damage. Written informed consent was obtained from all patients who participated in the study. This study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University and was carried out in accordance with the Declaration of Helsinki.

Percutaneous ultrasound-guided puncture biopsy of the right liver was performed in all PBC patients, followed by histopathological examination and pathological stage identification[23]. Four stages were defined based on intrahepatic bile duct injury[24]: Stage I: Cholangitis stage, chronic inflammation in the interlobular and septal bile duct. Lymphocytes and plasma cells around the damaged bile ducts infiltrate or form granuloma, but the inflammation in the portal area does not involve the liver parenchyma and there is no cholestasis; stage II: Periportal inflammation stage, with a continuous reduction in the number of interlobular bile ducts, reactive hyperplasia of bile ducts around the portal area, inflammation involving adjacent liver parenchyma and destruction of liver cells, and common focal necrosis, cholestasis also occurs; stage III: Progressive fibrosis stage, the portal area is continuously enlarged by inflammation and fibrosis progression, the fibrous septa formed gradually widens, and cholestasis is aggravated; stage IV: Liver cirrhosis stage, fibrous septa divides the liver parenchyma into patchy nodules, regenerating nodules, and forming pseudo lobules.

### Extraction of total RNA from plasma samples and cells

The relative expression levels of S100A6 and lncRNAs in plasma were measured in PBC patients, as well as human intrahepatic biliary epithelial cell lines. Total RNA was extracted from plasma and HiBECs by an RNA extraction kit (Bioteke, China), according to the manufacturer's instructions.

### Reverse transcription and quantitative PCR for S100A6 and lncRNAs

Total RNA was amplified by reverse transcription using a reverse transcription kit (PrimeScript™ RT Master Mix, TaKaRa, China)[25]. All reactions were completed in a

Themocycler (Mastercycler nexus, Eppendorf, Germany). Then, quantitative PCR was performed using SYBR® Premix Ex TaqTM II kit (Takara, China) on the LightCycler 480 (Roche, Germany). GAPDH was used as an internal reference, and served as an internal control for plasma RNA quality. S100A6 and lncRNAs expression were calculated by the $2^{-\Delta\Delta Ct}$ method {$2^{[(\text{Mean ct of RNA–mean ct of GAPDH}) - (\text{mean ct of control–mean ct of GAPDH})]}$}[26]. The calculated result was the relative quantitative expression value of S100A6 and lncRNAs compared with the internal reference. Primers for reactions were designed by Primer Premier 6.0 (Canada) software (Table 1)[27].

### Statistical analysis

Statistical Package for Social Science 23.0 software (IBM Solutions Statistical Package for the Social Sciences Incorporated, USA) and GraphPad Prism 8 (GraphPad Software, Incorporated, San Diego, CA, USA) were used for all statistical analyses. The normal distribution data were recorded (mean ± SD), and comparisons between the two groups were performed using the unpaired *t*-test. The paired *t*-test was used to compare the expression levels before and after treatment. Non-normal distribution data were analyzed using the non-parametric Mann-Whitney *U* test[28]. Categorical data were analyzed using the $\chi^2$ test. The correlation between the plasma level of S100A6 mRNA and lncRNAs was analyzed using Pearson or Spearman correlation analysis. Receiver operating characteristic (ROC) curves were constructed and the areas under the curves (AUC) were used to evaluate the value of plasma S100A6 mRNA and lncRNAs as biomarkers for the diagnosis and staging of PBC[29]. $P < 0.05$ was considered statistically significant.

## RESULTS

### Identification of the target gene

"GEO2R" was used to analyze the differentially expressed genes in liver tissues of BDL and sham mice of GSE29776. The top 10 up- and down-regulated genes of GSE29776 in the BDL and sham group are listed in Table 2. To identify potential biomarkers for PBC diagnosis and staging, we used qRT-PCR to validate the analysis of bioinformatics up-regulated genes in plasma of 30 PBC patients and 30 healthy controls. It was found that S100A6 showed the greatest change in the plasma of PBC patients ($t = 20.28$, $P < 0.0001$) (Figure 2). Therefore, S100A6 was selected as the target gene in this study.

### Expression of S100A6 protein in the BDL mouse model

HE staining revealed histological changes in liver tissues, with the BDL group showing liver cell swelling, vacuolar degeneration, and coagulative necrosis. Inflammatory cell infiltration was observed in the portal area and around the bile duct, and fibrosis around the bile duct (Figure 3A-C). In the sham group, there was no or minimal inflammatory cell infiltration around the portal area and bile duct (Figure 3D-F).

Double immunofluorescence staining was used to label CK19 and S100A6 proteins. A fluorescence microscope was used for observation and Image J software was used for graph analysis. The results showed that S100A6 labeled with fluorescein isothiocyanate showed emerald green fluorescence and CK19 labeled with tetraethyl rhodamine isothiocyanate showed red fluorescence. Red and green fluorescent overlapping images showed that CK19 and S100A6 proteins were positively expressed in the iBECs of BDL mice (Figure 4A-C), while these two proteins were weakly expressed in the iBECs of mice in the sham group (Figure 4D-F).

### The expression of S100A6 and lncRNAs analyzed in HiBECs

To investigate the mechanism of S100A6 and lncRNAs, the expression of S100A6 and lncRNAs was studied in HiBECs. Normal and HiBECs treated with GCDC were detected by qRT-PCR. The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in HiBECs treated with GCDC compared with controls (2.97 ± 0.43 *vs* 1.09 ± 0.08, $P = 0.0018$; 2.70 ± 0.26 *vs* 1.10 ± 0.10, $P = 0.0006$; 2.23 ± 0.21 *vs* 1.10 ± 0.10, $P = 0.0011$; 1.20 ± 0.04 *vs* 3.03 ± 0.15, $P < 0.0001$, respectively) (Figure 5).

### Demographics and clinical features of PBC patients compared with healthy controls

There were no differences in age and gender between the training set and validation

**Table 1 Primer sequences used in this study**

| Target gene | Forward sequence (5'→3') | Reverse sequence (5'→3') |
|---|---|---|
| *S100A6* | AATGTGCGTTGTGTAAGC | CGGTCCAAGTCTTCCATC |
| *LINC00312* | GGAAGGAATACCACAGAAGT | TGAAGAACAGGACATTGACA |
| *LINC00472* | AGAGTTGCTGTAGAAGAAGG | AGGAGGAGAGTAGAAGAGAC |
| *LINC01257* | TGCTGCGAATGATGACTT | AGGACTTGAATCTGCTACTG |
| *HMGB2* | TTACGTTCCTCCCAAAGGTG | TCTTTGGCTGACTGCTCAGA |
| *RC3H2* | TTGCAAAGAAATGCGTTGAG | GATTGGCAGACAACTGCTGA |
| *ADAMTS1* | CCTCTGTCTGTGTGCAAGGA | GTGGCTCCAGTTGGAATTGT |
| *SERPINE1* | CTCTCTCTGCCCTCACCAAC | GTGGAGAGGCTCTTGGTCTG |
| *PALD1* | GCCGAAGTTGTTCCCATTTA | GCTGAAAGTCAGAGCCAACC |
| *GSTA4* | TCCGTGAGATGGGTTTTAGC | TGCCAAAGAGATTGTGCTTG |
| *ACTA2* | TTCAATGTCCCAGCCATGTA | GAAGGAATAGCCACGCTCAG |
| *GAPDH* | GCACCGTCAAGGCTGAGAAC | TGGTGAAGACGCCAGTGGA |

ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSTA4: Glutathione S-transferase alpha 4; HMGB2: High mobility group box 2; PALD1: Phosphatase domain containing paladin 1; RC3H2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; SERPINE1: Serpin family E member 1.

set ($P$ = 0.504 and $P$ = 1.0, respectively, Table 3). Moreover, there were no differences in age and gender between PBC patients and healthy controls ($P$ = 0.58 and $P$ = 1.0, respectively). Clinical serological data including alanine aminotransferase, aspartate aminotransferase, ALP, gamma-glutamyl transpeptidase, total bilirubin, direct bilirubin, total bile acid, hyaluronic acid, laminin, collagen type IV (C-IV) and procollagen III were all significantly higher in PBC patients than in healthy controls ($P$ < 0.0001, Table 4).

### *Differential expression of S100A6 and lncRNAs in PBC patients compared with HCs*

To compare the mean expression levels of S100A6 mRNA, LINC00312, and LINC01257 in PBC patients and healthy controls, the Kolmogorov–Smirnov test was used to check normality. The results showed that these variables had a normal distribution ($P$ > 0.05), and the $t$-test was used for analysis, as the relative expression of LINC00472 showed a skewed distribution, and was normally distributed after logarithmic conversion based on 10. The results showed that the expression levels of S100A6 mRNA, log10 LINC00472 and LINC1257 in PBC patients were significantly up-regulated compared to the healthy controls (3.01 ± 1.04 *vs* 2.09 ± 0.87; 2.46 ± 1.03 *vs* 1.77 ± 0.84; 3.49 ± 1.64 *vs* 2.37 ± 0.96, $P$ values were all less than 0.0001, Figure 6A, C and D). The mean expression level of LINC00312 was significantly lower in PBC plasma samples compared with HCs (1.70 ± 0.33 *vs* 2.07 ± 0.53, $P$ < 0.0001, Figure 6B).

### *Distribution of S100A6 and lncRNAs expression levels in different stages of PBC*

The unpaired $t$-test analysis of variance was performed to evaluate differences in the expression of S100A6 and lncRNAs among different PBC stages and healthy controls (Figure 7). The results showed that the mean expression of S100A6 in the advanced stage (III and IV) of PBC was up-regulated compared to that in HCs and the early stage (II) (3.38 ± 0.71 *vs* 2.09 ± 0.87, $P$ < 0.0001; 3.38 ± 0.71 *vs* 2.57 ± 1.21, $P$ = 0.0003, respectively); and in the early stage (II), it was higher than that in HCs (2.57 ± 1.21 *vs* 2.09 ± 0.87, $P$ = 0.03) (Figure 7A). The mean expression of LINC00312 in the advanced stage was lower than that in the early stage and HCs (1.39 ± 0.29 *vs* 1.56 ± 0.33, $P$ = 0.01; 1.39 ± 0.29 *vs* 2.07 ± 0.53, $P$ < 0.0001, respectively) (Figure 7B); in addition, the mean expression of LINC00312 in the early stage was lower than that in HCs (1.56 ± 0.33 *vs* 2.07 ± 0.53, $P$ < 0.0001) (Figure 7B). The mean expression of log10 LINC00472 in the advanced stage was higher than that in the early stage and HCs (2.99 ± 0.87 *vs* 1.81 ± 0.83, $P$ < 0.0001; 2.99 ± 0.87 *vs* 1.77 ± 0.84, $P$ < 0.0001, respectively) (Figure 7C). The mean expression of LINC01257 in both the early stage and advanced stage were up-regulated compared with HCs (3.88 ± 1.55 *vs* 2.37 ± 0.96, $P$ < 0.0001; 3.57 ± 1.79 *vs* 2.37 ± 0.96, $P$ < 0.0001, respectively) (Figure 7D).

**Table 2 Top 10 dysregulated genes in bile duct ligation and sham mice**

| Gene name | Transcript | Lg fold change |
|---|---|---|
| *Up-regulated* | | |
| *Hmgb2* | ENSMUSG00000054717 | 3.53 |
| *Rc3h2* | ENSMUSG00000075376 | 3.33 |
| *Adamts1* | ENSMUSG00000022893 | 3.15 |
| *Serpine1* | ENSMUSG00000037411 | 3.08 |
| *S100a6* | ENSMUSG00000001025 | 2.98 |
| *Pald1* | ENSMUSG00000020092 | 2.67 |
| *Gsta4* | ENSMUSG00000032348 | 2.50 |
| *D17H6S56E-5* | NM_033075 | 2.46 |
| *Acta2* | ENSMUSG00000035783 | 2.39 |
| *Ifi204* | ENSMUSG00000073489 | 2.33 |
| *Down-regulated* | | |
| *Mcm10* | ENSMUSG00000026669 | 3.23 |
| *Upp2* | ENSMUSG00000026839 | 2.85 |
| *2810043O03Rik* | AK012901.1 | 2.59 |
| *Dnaaf5* | ENSMUSG00000025857 | 2.41 |
| *Sva* | ENSMUSG00000023289 | 2.40 |
| *Naca* | ENSMUSG00000061315 | 2.35 |
| *Dhps* | ENSMUSG00000060038 | 2.33 |
| *Cdh15* | ENSMUSG00000031962 | 2.26 |
| *Gzmm* | ENSMUSG00000054206 | 2.20 |
| *Alox12* | ENSMUSG00000000320 | 2.15 |

2810043O03Rik: RIKEN complementary deoxyribonucleic acid 2810043O03 gene; Acta2: Actin alpha 2, smooth muscle; Adamts1: A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1; Alox12: Arachidonate 12-lipoxygenase; Cdh15: Cadherin 15; D17H6S56E-5: Deoxyribonucleic acid segment, Chr 17, human D6S56E 5; Dhps: Deoxyhypusine synthase; Dnaaf5: Dynein, axonemal assembly factor 5; Gsta4: Glutathione S-transferase alpha 4; Gzmm: Granzyme M (lymphocyte met-ase 1); Hmgb2: High mobility group box 2; Ifi204: Interferon activated gene 204; Mcm10: Minichromosome maintenance 10 replication initiation factor; Naca: Nascent polypeptide-associated complex alpha polypeptide; Pald1: Phosphatase domain containing paladin 1; Rc3h2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; Serpine1: Serpin family E member 1; Sva: Seminal vesicle antigen; Upp2: Uridine phosphorylase 2.

### *Diagnosis and staging value of plasma S100A6 and lncRNAs for PBC patients*

ROC curves were used to evaluate the potential diagnostic value of each biomarker for PBC. The AUC for S100A6, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.759, 0.7292, 0.6942 and 0.7158, respectively (Figure 8A-D). Furthermore, AUC for these four genes in PBC staging were 0.666, 0.661, 0.839 and 0.5549, respectively (Figure 8E-H).

Pearson or Spearman correlation analysis was performed to evaluate the correlation between relative expression of S100A6 mRNA and lncRNAs, as well as relative expression of S100A6 mRNA or lncRNAs and clinical serological data in PBC patients. Relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r$ = 0.683, $P$ < 0.0001); serum level of C-IV was positively correlated with relative expression of log10 LINC00472 ($r$ = 0.482, $P$ < 0.0001); relative expression of S100A6 mRNA was positively correlated with serum level of C-IV ($r$ = 0.732, $P$ < 0.0001) (Figure 9).

### *Comparison of expression levels of biomarkers before and after treatment*

A total of 58 PBC patients were followed up after their treatment for one year. Paired $t$-test analysis was used to compare the expression levels of these four genes before and after treatment. The relative expression of S100A6 mRNA, log10 LINC00472, and

**Table 3 Demographics and clinical characteristics in the training and validation datasets**

| Characteristics | Training | Validation | *P* value |
|---|---|---|---|
| No. | 140 | 115 | - |
| Age, mean ± SD, yr | 56.0 ± 13.9 | 57.2 ± 13.2 | 0.504 |
| Gender, *n* (%) | | | |
| Male | 17 (12.1) | 13 (11.3) | |
| Female | 123 (87.9) | 102 (88.7) | 1.0 |
| Pathological stage | | | |
| I and II | 36 (45.0) | 26 (40.0) | |
| III and IV | 44 (55.0) | 39 (60.0) | 0.614 |

Normally distributed data are expressed as mean ± SD. Categorical variable values are described as *n* (%). SD: Standard deviation.

**Table 4 Demographics and clinical characteristics of primary biliary cholangitis patients and healthy controls[1]**

| Characteristics | PBC (*n* = 145) | HCs (*n* = 110) | *P* value |
|---|---|---|---|
| Age, mean ± SD, yr | 56.1 ± 13.4 | 55.3 ± 11.8 | 0.58 |
| Gender, *n* (%) | | | |
| Male | 17 (12.5) | 13 (16.7) | |
| Female | 128 (87.5) | 97 (83.3) | 1.00 |
| ALT, U/L | 78.6 ± 35.7 | 18.4 ± 6.5 | < 0.001 |
| AST, U/L | 104.8 ± 43.5 | 20.2 ± 4.3 | < 0.001 |
| ALP, U/L | 257.4 ± 79.9 | 64.7 ± 14.5 | < 0.001 |
| γGT, U/L | 416.7 ± 209.2 | 26.3 ± 10.4 | < 0.001 |
| TBIL, μmol/L | 66.8 ± 10.6 | 11.8 ± 4.0 | < 0.001 |
| DBIL, μmol/L | 51.9 ± 11.4 | 6.4 ± 0.5 | < 0.001 |
| TBA, μmol/L | 71.3 ± 11.6 | 2.8 ± 0.4 | < 0.001 |
| HA, ng/mL | 146.9 (104.6-190.1) | 67.0 (53.9-79.7) | < 0.001 |
| LN, ng/mL | 148.9 (76.7-182.8) | 70.4 (58.7-82.9) | < 0.001 |
| C-IV, ng/mL | 154.8 (121.1-192.0) | 60.1 (55.2-66.7) | < 0.001 |
| PC-III, ng/mL | 161.0 (135.1-184.5) | 57.3 (49.9-63.5) | < 0.001 |
| Pathological stage | | | |
| I and II | 62 (42.8) | - | |
| III and IV | 83 (57.2) | - | |

[1]Normally distributed data are expressed as means ± SD, variables with a skewed distribution are presented as median (interquartile range). Categorical variable values are described as *n* (%). γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBIL: Direct bilirubin; HA: Hyaluronic acid; LN, Laminin; PC-III: Procollagen III; TBA: Total bile acid; TBIL: Total bilirubin; SD: Standard deviation; PBC: Primary biliary cholangitis; HCs: Healthy controls.

LINC01257 were significantly decreased after treatment (2.35 ± 1.02 *vs* 3.06 ± 1.04, $P$ = 0.0018; 1.99 ± 0.83 *vs* 2.33 ± 0.96, $P$ = 0.036; 2.84 ± 0.92 *vs* 3.69 ± 1.54, $P$ = 0.0006, respectively); in addition, the relative expression of LINC00312 increased significantly after treatment compared with before treatment (1.95 ± 0.35 *vs* 1.73 ± 0.32, $P$ = 0.0007) (Figure 10).

### *Differences between PBC patients with high and low levels of LINC00472*

According to ROC curves analysis, the AUC of log10 LINC00472 was 0.839 ($P$ < 0.0001)

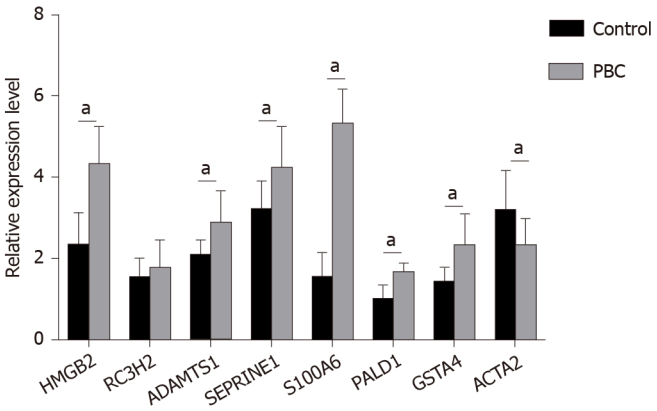


**Figure 2 Validation of top 10 up-regulated genes in the plasma of primary biliary cholangitis patients and healthy controls by reverse transcriptase polymerase chain reaction.** $^{a}P < 0.0001$. ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif 1; GSTA4: Glutathione S-transferase alpha 4; HMGB2: High mobility group box 2; PALD1: Phosphatase domain containing paladin 1; RC3H2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; SERPINE1: Serpin family E member 1; PBC: Primary biliary cholangitis.

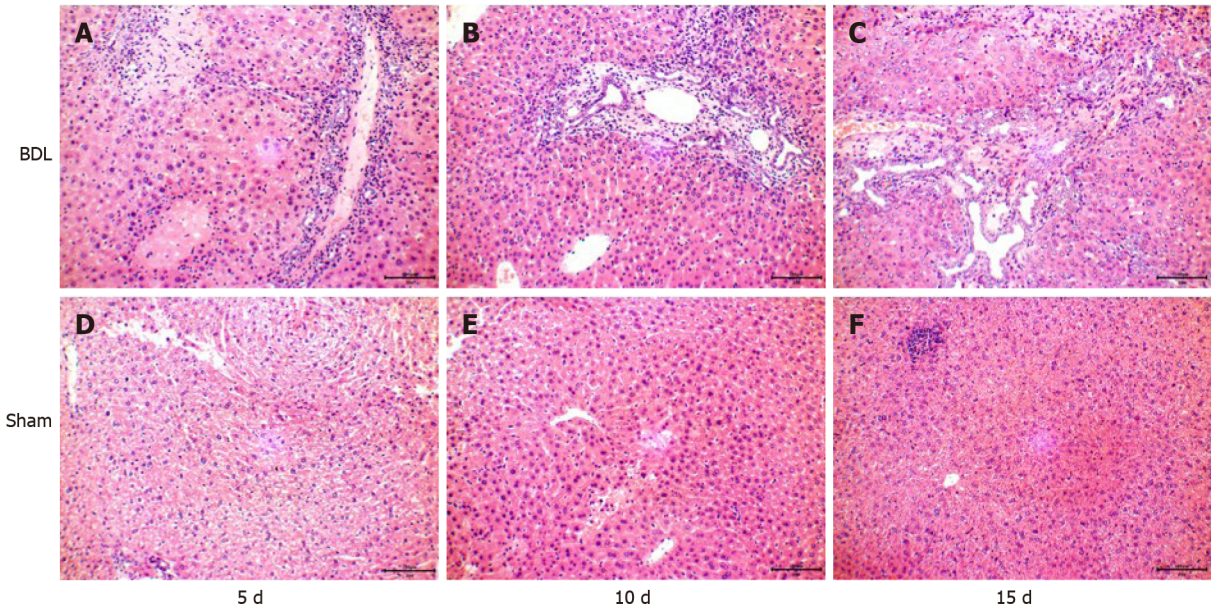


**Figure 3 Liver tissues of mice in the bile duct ligation group and sham group were observed under an optical microscope (hematoxylin-eosin stain, × 400).** A-C: 5, 10 and 15 d after surgery in the bile duct ligation group, respectively; D-F: 5, 10 and 15 d after surgery in the sham group, respectively. BDL: Bile duct ligation.

and the Youden index was 1.551. Accordingly, the patients in the PBC group were divided into L1 (log10 LINC00472 < 2.33) and L2 (log10 LINC00472 ≥ 2.33) subgroups. The baseline characteristics of PBC patients classified by the relative expression of the log10 LINC00472 cutoff value (2.33) is shown in Table 5. The relative expression of S100A6 mRNA and serum level of C-IV were lower in the L1 subgroup ($P < 0.0001$, Table 5); in addition, the relative expression of LINC01257 was higher in the L1 subgroup compared to the L2 subgroup ($P = 0.005$, Table 5).

### *Validation of diagnosis and staging value*

The parameters estimated from the training data set were used to predict the probability of being diagnosed with PBC and staging of PBC for the independent validation data set. ROC curves were also constructed to predict the probability of diagnosis and staging. The AUC of S100A6 mRNA, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.769, 0.772, 0.755 and 0.695, respectively (Figure 11A-D). Moreover, the AUC for log10 LINC00472 in PBC staging was 0.835 (Figure 11E).

**Table 5 Characteristics of primary biliary cholangitis patients based on the expression of log10 LINC00472 cutoff value**

| | Relative Expression of log10 LINC00472 | | |
|---|---|---|---|
| | L1 (< 2.33) (*n* = 38) | L2 (≥ 2.33) (*n* = 42) | *P* value |
| Age, mean ± SD, years | 60.3 ± 14.9 | 55.1 ± 14.0 | 0.109 |
| Gender, *n* (%) | | | |
| Male | 5 (13.2) | 5 (11.9) | |
| Female | 33 (86.8) | 37 (88.1) | 0.886 |
| ALT, U/L | 73.2 (52.7-100.1) | 73.2 (46.2-100.7) | 0.985 |
| AST, U/L | 109.2 ± 45.9 | 103.0 ± 44.6 | 0.543 |
| ALP, U/L | 264.0 ± 89.4 | 252.2 ± 78.3 | 0.532 |
| γGT, U/L | 420.2 ± 197.9 | 413.2 ± 237.1 | 0.887 |
| TBA, μmol/L | 73.2 ± 12.4 | 70.9 ± 13.3 | 0.438 |
| TBiL, μmol/L | 68.0 (63.0-73.0) | 63.0 (58.0-73.0) | 0.166 |
| DBiL, μmol/L | 50.6 ± 9.8 | 52.2 ± 11.4 | 0.505 |
| LINC00312 | 1.51 ± 0.32 | 1.43 ± 0.31 | 0.261 |
| S100A6 | 2.40 ± 1.05 | 3.57 ± 0.66 | < 0.0001 |
| LINC01257 | 4.25 ± 1.39 | 3.22 ± 1.78 | 0.005 |
| HA, ng/mL | 144.8 (101.6-208.8) | 135.5 (95.4-195.4) | 0.537 |
| LN, ng/mL | 126.1 (48.4-178.4) | 156.1 (57.6-175.8) | 0.78 |
| C-IV, ng/mL | 127.2 (100.9-170.4) | 176.0 (154.7-232.0) | < 0.0001 |
| PC-III, ng/mL | 156.6 (125.8-190.1) | 161.6 (128.0-184.5) | 0.916 |

SD: Standard deviation; γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBIL: Direct bilirubin; HA: Hyaluronic acid; LN: Laminin; PC-III: Procollagen III; S100A6: S100 calcium binding protein A6; TBA: Total bile acid; TBIL: Total bilirubin.

## DISCUSSION

PBC is a type of cholestatic liver disease which is a pathophysiological process caused by the obstruction of bile secretion and excretion. After analyzing the expression levels of the top 10 up-regulated genes of GSE29776 in the plasma of PBC patients, it was found that the difference in S100A6 mRNA expression levels between PBC patients and healthy controls was greatest ($t$ = 20.28, $P$ < 0.0001). Therefore, S100A6 was selected as the target gene. BDL is a common procedure for biliary obstruction widely used in rodent models of cholestasis and liver damage[30]. Immunofluorescence double labeling analysis was performed to identify the overexpression of S100A6 protein in the intrahepatic bile duct epithelial cells of BDL mice compared with sham mice, which verified the results predicted by bioinformatics analysis. In this study, the bile duct cells proliferated greatly in the liver tissue 10 d after the operation in the BDL group[31,32], and S100A6 protein was expressed in large quantities during the corresponding period. However, the number of bile duct cells in the sham group was relatively low, and the expression of S100A6 protein was also relatively low. Therefore, it can be seen that proliferation of bile duct cells was specifically enhanced when cholestatic liver injury occurred; thus, there was a difference in S100A6 between the two groups. S100A6 is expressed as a 89-amino acid protein in mice and rats, a 90-amino acid protein in humans and rabbits, and subtypes A (92 amino acids) and B (91 amino acids) in chickens, which may be produced by mRNA selective splicing[33]. In this study, the S100A6 antibodies used were universal in humans and mice, so the results of the BDL mouse model could indirectly reflect the up-regulation of S100A6 expression in human intrahepatic cholestasis.

In this study, S100A6 mRNA was overexpressed in the plasma of PBC patients compared with healthy controls. S100A6 expression is up-regulated in breast cancer, thyroid cancer, colorectal cancer, various types of skin tumors, acute myelogenous leukemia, epithelial tissues and other highly proliferating cell lines[34]. Apoptosis in

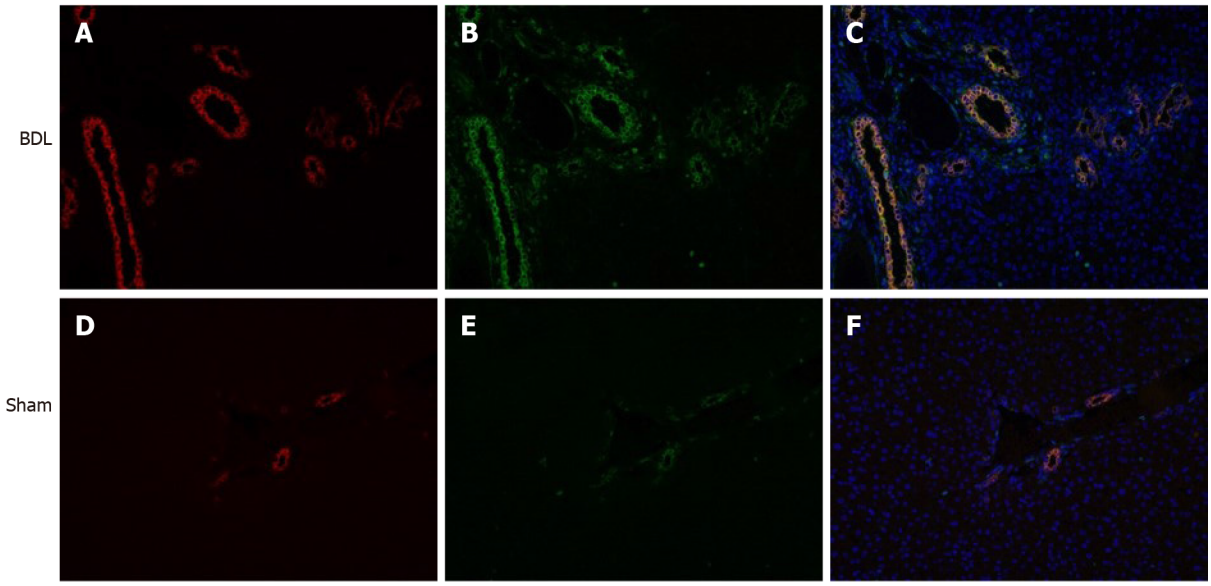


**Figure 4 C57BL/6J mouse liver tissue double immunofluorescence (red light: Cytokeratin 19 protein, green light: S100 calcium binding protein A6 protein, × 200).** A: Cytokeratin 19 (CK19) protein in bile duct ligation (BDL) mouse; B: S100 calcium binding protein A6 protein (S100A6) protein in BDL mouse; C: CK19 and S100A6 proteins merge in BDL mouse; D: CK19 protein in sham mouse; E: S100A6 protein in sham mouse; F: CK19 and S100A6 proteins merge in sham mouse. BDL: Bile duct ligation.

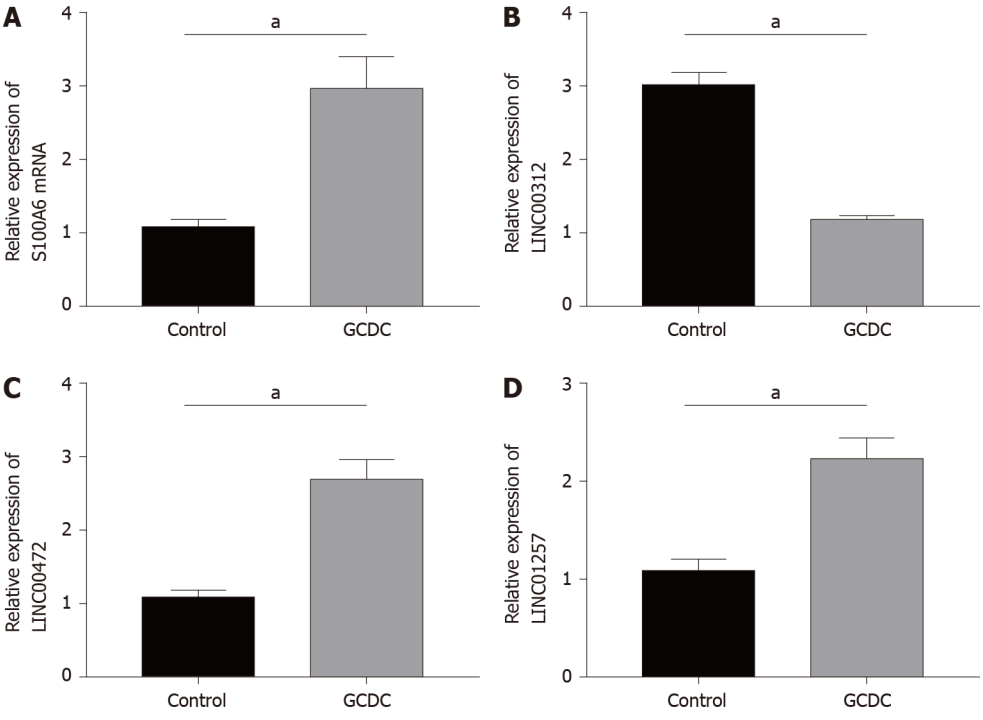


**Figure 5 The expression of S100 calcium binding protein A6 protein messenger ribonucleic acid, LINC00312, LINC00472 and LINC01257 in human intrahepatic biliary epithelial cells (control and treated with glycochenodeoxycholate) analyzed by quantitative reverse transcription-polymerase chain reaction.** A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: LINC00472; D: LINC01257. $^{a}P < 0.005$. GCDC: Glycochenodeoxycholate.

PBC is considered to be the cell effector injury mediated by T cells. Changes in apoptosis and apoptosis-related molecular expression of bile duct cells have been reported in bile duct lesions, but immune-mediated injury of bile duct epithelial cells has not been fully elucidated[35]. Joo *et al*[36] found that S100A6 may be involved in the process of apoptosis by regulating the transcriptional regulation of caspase-3. Therefore, it seems that S100A6 may play an important role in the pathogenesis of PBC.

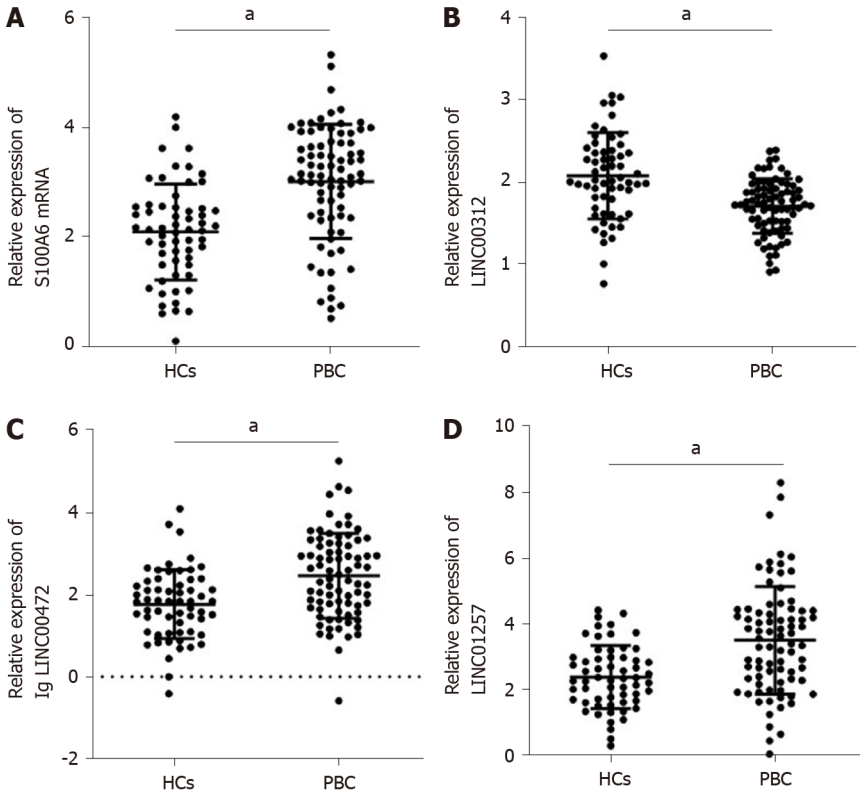


**Figure 6 Differential expression of plasma s100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids in primary biliary cholangitis plasma samples compared with healthy controls.** A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257. [a]*P* < 0.0001. HCs: Healthy controls; PBC: Primary biliary cholangitis.

The expression of lncRNAs is not only closely related to the occurrence and development of tumors[37], but also associated with autoimmune diseases[38]. In this study, the expression of lncRNAs selected by bioinformatics analysis was differentially expressed in the plasma of PBC patients compared with healthy controls. The levels of plasma LINC00312 was significantly down-regulated in PBC patients, while LINC00472 and LINC01257 were up-regulated in PBC patients, indicating that these lncRNAs might be valuable for PBC diagnosis. ROC curves were used to evaluate the diagnostic value of each marker. The differential expression in plasma between PBC patients and heathy controls indicated that S100A6 mRNA (AUC = 0.76, $P < 0.0001$), LINC00312 (AUC = 0.73, $P < 0.0001$), log10 LINC00472 (AUC = 0.69, $P < 0.0001$) and LINC01257 (AUC = 0.72, $P < 0.0001$) may be potential biomarkers for the diagnosis of PBC.

Furthermore, the ROC curves analysis also showed that plasma S100A6 mRNA (AUC = 0.67, $P = 0.01$), LINC00312 (AUC = 0.66, $P = 0.01$) and log10 LINC00472 (AUC = 0.84, $P < 0.0001$) could also be used to predict disease progression in PBC. In particular, LINC00472 had high diagnostic value for PBC staging (sensitivity was 77.27%, specificity was 77.78%). According to the cutoff value (2.33) of log10 LINC00472, the relative expression of S100A6 mRNA and serum level of C-IV in the high-level group were higher than those in the low-level group.

LINC00312, also known as NAG7, was found to inhibit proliferation and induce apoptosis in nasopharyngeal carcinoma (NPC) cells but also stimulate NPC cell invasion. LINC00312 was significantly down-regulated in NPC tissues compared with non-cancerous nasopharyngeal epithelium tissues. Positive expression of LINC00312 was negatively correlated with tumor size but positively correlated with lymph node metastasis[39]. High expression of LINC00472 was associated with less aggressive breast tumors and better prognosis. Patients with high expression of LINC00472 had a significantly reduced risk of recurrence and death compared to those with low expression. Patients with high expression of LINC00472 also responded better to adjuvant chemotherapy or hormone therapy than those with low expression[40]. Therefore, studies on S100A6, LINC00312 and LINC00472 have all been related to tumors. This study is the first to explore the relationship between these three genes and autoimmune diseases. In addition, we investigated the relationship between the expression of LINC01257 and diseases for the first time.

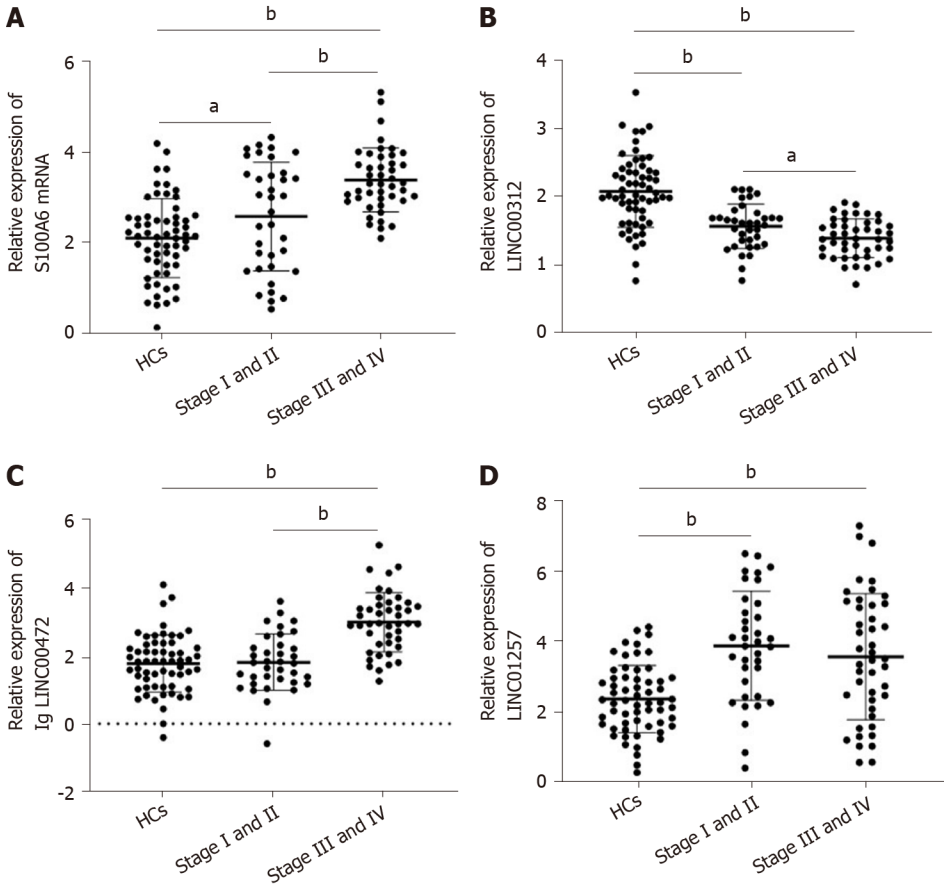


**Figure 7 Scatter plot and distribution of expression levels of s100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids in different stages of primary biliary cholangitis compared with healthy controls.** The unpaired *t*-test analysis of variance was performed to examine differences in S100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids expression levels between various groups. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257. $^{a}P < 0.05$, $^{b}P < 0.0001$. HCs: Healthy controls.

The expression levels of plasma S100A6, LINC00312, LINC00472 and LINC01257 in PBC patients before and after treatment were analyzed by the paired *t*-test. It was found that the elevated biomarkers decreased after treatment, while the reduced biomarker increased. This provides further evidence that these four genes are biomarkers for PBC diagnosis.

The correlation analysis showed that relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r = 0.683$, $P < 0.0001$) and the serum level of C-IV ($r = 0.732$, $P < 0.0001$). C-IV serves as a histochemical marker of perisinusoidal basement membrane formation in liver disease[41]. It was further illustrated that S100A6 may be associated with PBC liver injury. The relative expression of log10 LINC00472 was positively correlated with the serum level of C-IV ($r = 0.482$, $P < 0.0001$), indicating that it was related to the disease severity of PBC. It was suggested that LINC00472 can be used as a marker of PBC staging. However, in our study, the four biomarkers did not correlate with the cholestasis indicator ALP, and we think this may be due to the following reasons: (1) The S100A6 protein was expressed in large quantities during the early period of cholestasis. This process may precede the increase in serum ALP level; (2) Proliferation of bile duct cells is characterized by irregular proliferation of intrahepatic bile ducts not only confined to portal areas, but also sprouting into periportal and parenchymal regions. This implies that the newly formed bile ducts are functionally ineffective[42,43]; and (3) In the late stage of liver fibrosis, considerable hepatocyte necrosis occurs.

Hepatocytes exposed to bile acids have been used in many studies on PBC. The most commonly used bile acid is GCDC, which is a type of toxic hydrophobic bile acid and can induce apoptosis of iBECs, form apoptotic bodies, and can lead to the pyruvate dehydrogenase complex E2 subunit as an autoimmune antigen to be exposed. A series of immune responses are then activated[44]. Hisamoto *et al*[45] studied the effects of hydrophobic bile acid on human BECs and autologous spleen mononuclear cells, especially the effects of GCDC on anion exchange protein

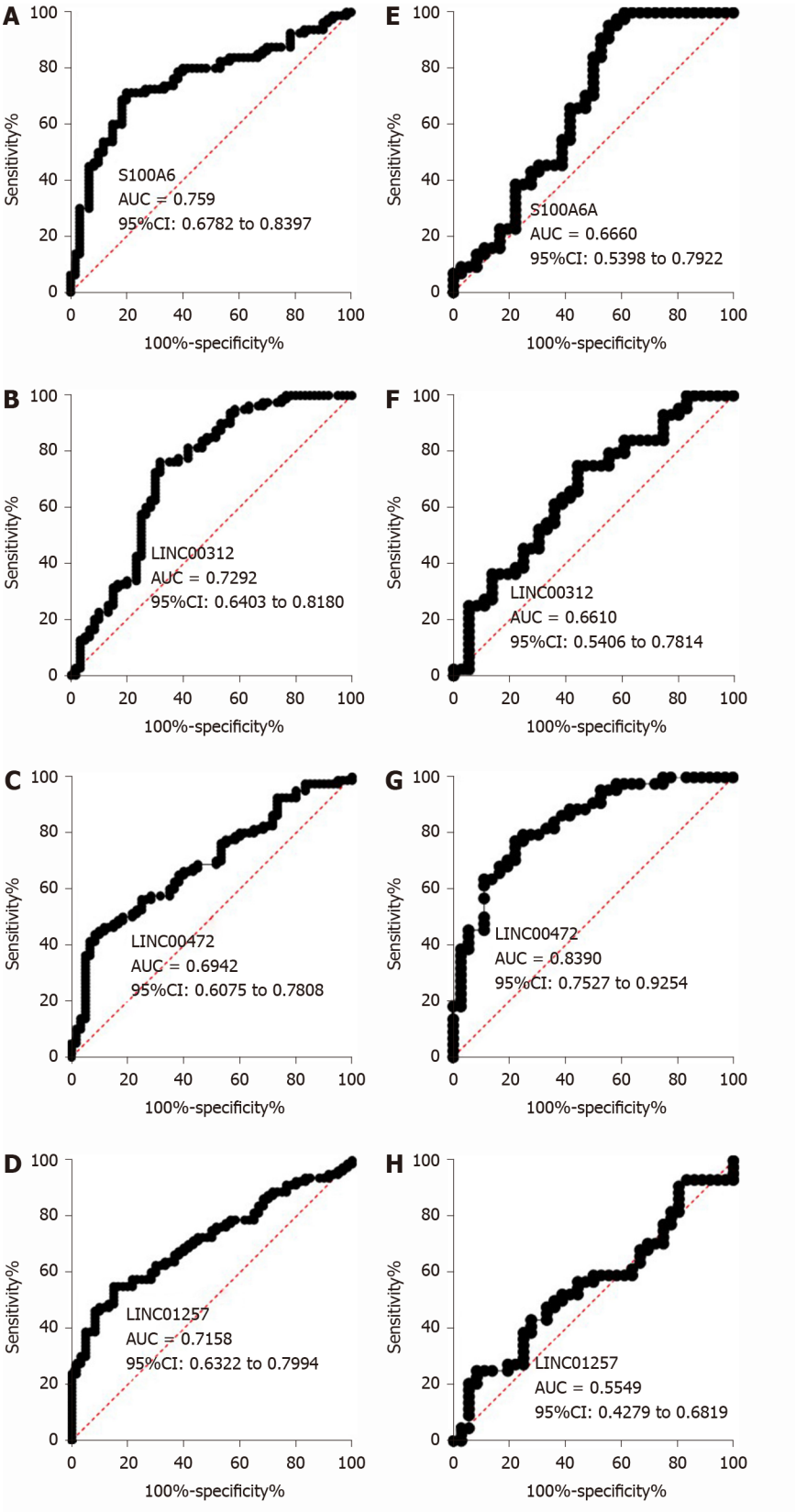


**Figure 8 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the training set.** A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the training set; E-H: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis staging in the training set. AUC: Area under the curve; CI: Confidence interval.

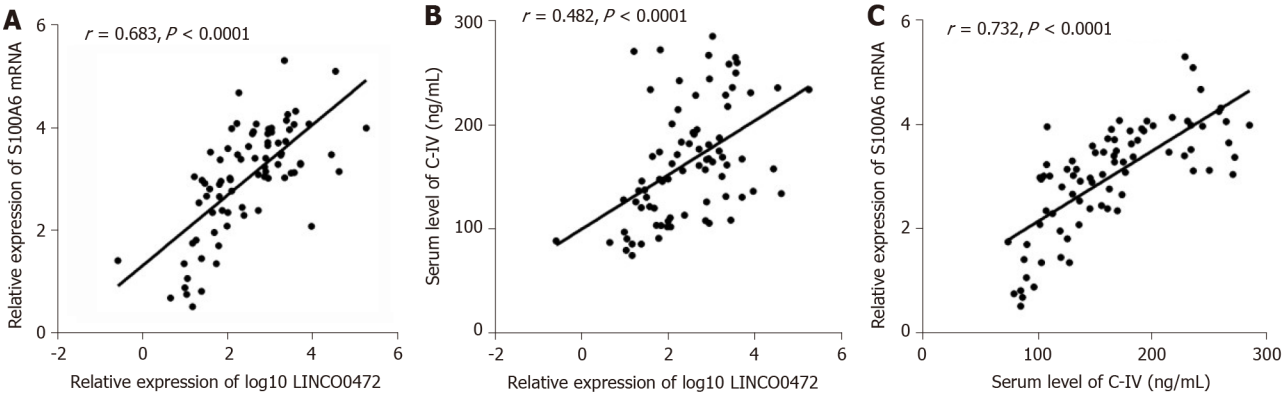


**Figure 9 Correlation analysis of biomarkers and clinical serological indices.** A: The positive correlation between relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid and log10 LINC00472, $r = 0.683$, $P < 0.0001$; B: The positive correlation between relative expression of log10 LINC00472 and serum level of collagen type IV, $r = 0.482$, $P < 0.0001$; C: The positive correlation between serum level of collagen type IV and relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid, $r = 0.732$, $P < 0.0001$.

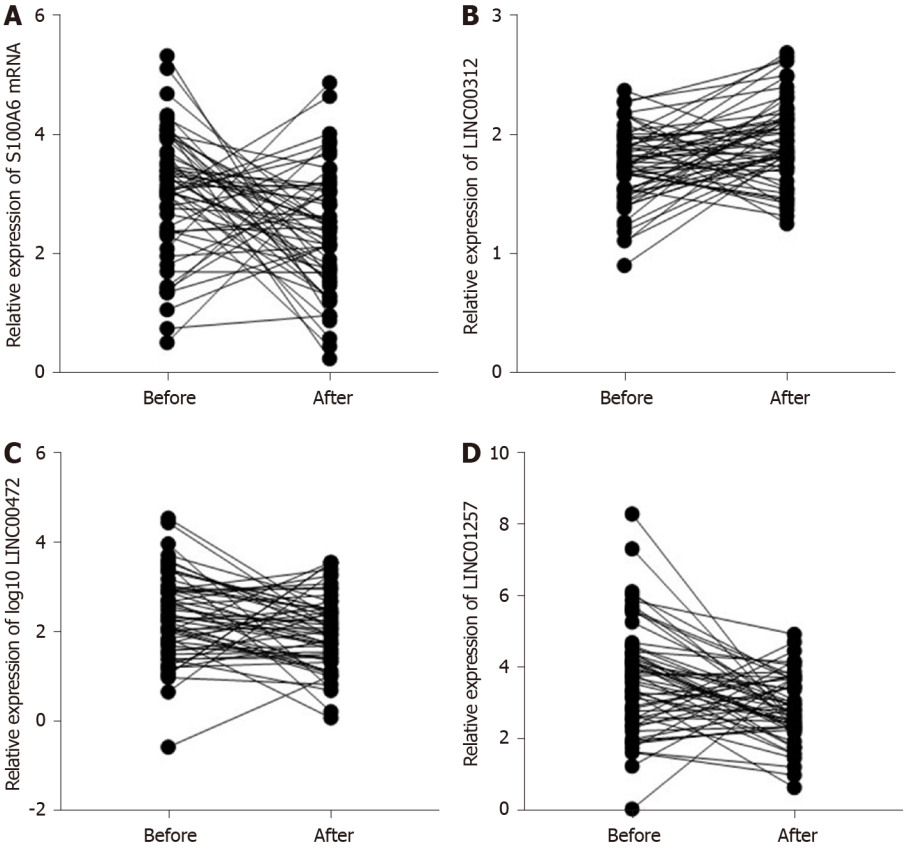


**Figure 10 Comparison and analysis of s100 calcium binding protein A6 protein messenger ribonucleic acid, LINC00312, log10 LINC00472, LINC01257 expression levels in primary biliary cholangitis patients before and after treatment using the paired *t*-test.** A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257.

expression of BECs and on the phenotype of BECs and local inflammatory response. It was proved that GCDC reduced the expression of anion exchange in BECs and accelerated the aging of BECs by inducing reactive oxygen species. Therefore, this study used GCDC to treat HiBECs to simulate a cholestatic environment and assess its damage to HiBECs. In this study, the expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in GCDC-treated HiBECs compared with controls, consistent with the expression in plasma of PBC patients. It was further proved that these four indicators are related to PBC diagnosis and staging.

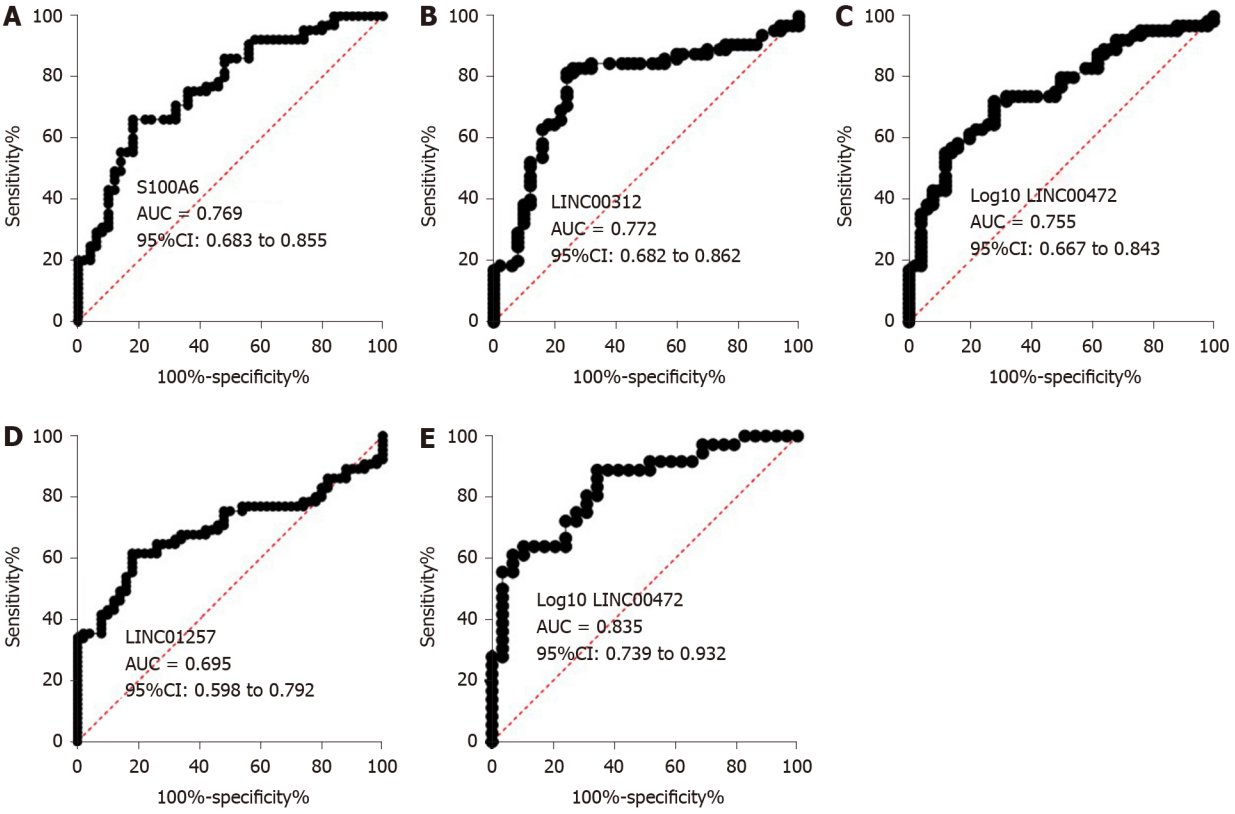


**Figure 11 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the validation set.** A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the validation set; E: Receiver operating characteristic curves of LINC00472 for primary biliary cholangitis staging in the validation set. AUC: Area under the curve; CI: Confidence interval.

The value of the above four biomarkers should be validated in an additional cohort of PBC patients and their specificity needs to be examined in other patient populations[46]. We chose another PBC cohort as the validation set. The AUC of the four genes were close to those in the training set. Therefore, the value of these four biomarkers in the diagnosis and staging of PCB was validated. However, in China, the vast majority are Han Chinese; therefore, it is difficult to verify these findings in other ethnic groups.

## CONCLUSION

In conclusion, the expression of S100A6 protein in BDL mice was up-regulated, the expression of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated, while LINC00312 was down-regulated both in the plasma of PBC patients and HiBECs treated with GCDC compared with controls. Although our study was confined to the expression analysis of S100A6 mRNA, LINC00312, LINC00472 and LINC01257, warranting further studies to investigate the mechanisms underlying the functional role of these four markers, nevertheless their potential as biomarkers for diagnosis and staging of PBC was elucidated by multiple evaluations in this study.

## ARTICLE HIGHLIGHTS

### *Research background*

Primary biliary cholangitis (PBC) is an autoimmune liver disease that mostly affects women. Fatigue and persistent pruritus are the most obvious symptoms. PBC may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells is the key to investigating the pathogenesis of PBC, but the accurate relationship between cholestasis and liver

fibrosis is still indistinct.

### Research motivation

To explore the target genes of intrahepatic biliary epithelial cell injury in PBC. To search for plasma biomarkers for early diagnosis and staging of PBC. To lay a foundation for further study on the pathogenesis of PBC.

### Research objectives

To explore the potential diagnosis and staging value of plasma S100 calcium binding protein A6 (S100A6) messenger ribonucleic acid (mRNA), LINC00312, LINC00472, and LINC01257 in primary biliary cholangitis.

### Research methods

The up-regulation of S100A6 was identified by double immunofluorescence in a bile duct ligation mouse model. We used quantitative reverse transcription-polymerase chain reaction to analyze the relative expression levels of S100A6 mRNA, long noncoding ribonucleic acids (lncRNAs) LINC00312, LINC00472 and LINC01257 both in patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

### Research results

The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in both the plasma of patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

### Research conclusions

These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a biomarker for staging in PBC.

### Research perspectives

Although we have demonstrated that S100A6 and related lncRNAs may be biomarkers for the diagnosis and staging of PBC, their detailed value needs to be analyzed in a large sample. The specific mechanisms of S100A6 and lncRNAs require further investigation.

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ORIGINAL ARTICLE

Basic Study

# Long non-coding RNA TP73-AS1 promotes pancreatic cancer growth and metastasis through miRNA-128-3p/GOLM1 axis

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## Abstract

***BACKGROUND***

Previous studies have suggested that long non-coding RNAs (lncRNA) TP73-AS1 is significantly upregulated in several cancers. However, the biological role and clinical significance of TP73-AS1 in pancreatic cancer (PC) remain unclear.

***AIM***

To investigate the role of TP73-AS1 in the growth and metastasis of PC.

***METHODS***

The expression of lncRNA TP73-AS1, miR-128-3p, and *GOLM1* in PC tissues and cells was detected by quantitative real-time polymerase chain reaction. The bioinformatics prediction software ENCORI was used to predict the putative binding sites of miR-128-3p. The regulatory roles of TP73-AS1 and miR-128-3p in cell proliferation, migration, and invasion abilities were verified by Cell Counting Kit-8, wound-healing, and transwell assays, as well as flow cytometry and Western blot analysis. The interactions among TP73-AS1, miR-128-3p, and GOLM1 were explored by bioinformatics prediction, luciferase assay, and Western blot.

***RESULTS***

The expression of TP73-AS1 and miRNA-128-3p was dysregulated in PC tissues and cells. High TP73-AS1 expression was correlated with a poor prognosis. TP73-AS1 silencing inhibited PC cell proliferation, migration, and invasion *in vitro* as well as suppressed tumor growth *in vivo*. Mechanistically, TP73-AS1 was validated to promote PC progression through GOLM1 upregulation by competitively binding to miR-128-3p.

***CONCLUSION***

Our results demonstrated that TP73-AS1 promotes PC progression by regulating

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the miR-128-3p/GOLM1 axis, which might provide a potential treatment strategy for patients with PC.

**Key Words:** Pancreatic cancer; Long non-coding RNA; TP73-AS1; miR-128-3p; GOLM1



**Core Tip:** In this study, the expression level of TP73-AS1 in pancreatic cancer (PC) was measured and its clinical significance was assessed. *In vitro* and *in vivo* experiments were performed to determine the roles of TP73-AS1 in the progression and development of PC. Moreover, the underlying molecular mechanisms were also illustrated, which could provide a novel therapeutic target for patients with PC.

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## INTRODUCTION

Pancreatic cancer (PC) is the fourth most frequent cause of cancer-related deaths with an extremely poor prognosis, especially in patients with advanced-stage PC [1,2]. Although standard treatments have been improved in recent years, the effectiveness of these treatments was still limited and surgical resection was the only chance to obtain curative treatment[2,3]. Hence, it is necessary to seek for new treatment to optimize therapeutic approaches.

Long non-coding RNAs (lncRNAs) are small endogenous non-coding RNAs whose lengths are larger than 200 nucleotides. LncRNAs have the capacity to regulate various biological processes such as tumor initiation, growth, metastasis, chemoresistance, and radioresistance by directly binding to partially complimentary sequences in their target genes[4-7]. Moreover, emerging evidence has revealed that lncRNAs could play crucial roles in the progression of PC[8,9]. LncRNA-BX111 was upregulated in pancreatic cancer and high BX111 expression was correlated with advance tumor-node-metastasis (TNM) stage, lymphatic invasion, and distant metastasis, as well as poor clinical prognosis in patients with PC[10]. Further investigation revealed that BX111 contributed to metastasis and progression of PC by regulating expression of ZEB1 and its downstream proteins E-cadherin and MMP2[10]. PVT1 was identified as a regulator of gemcitabine sensitivity with a genome-wide and piggyBac transposon-based genetic screening platform[11]. Therefore, lncRNAs may be new biological markers for disease diagnosis and could be taken as new drug targets, which would provide a new strategy for PC.

Dysregulation of TP73-AS1 has been identified in several human cancer types, including glioma, hepatocellular carcinoma, and non-small cell lung cancer[12-14]. However, little is known about the expression pattern and biological roles of TP73-AS1 in PC. In this study, the expression level of TP73-AS1 in PC was measured and its clinical significance was assessed. *In vitro* and *in vivo* experiments were performed to determine the roles of TP73-AS1 in the progression and development of PC. Further investigation indicated that the 3′ untranslated region (UTR) of *GOLM1* harbors a functional response element for miR-128-3p. Besides, miR-128-3p-3p could abrogate TP73-AS1-mediated expression of GOLM1, which suggested that TP73-AS1 could act as a molecular sponge to decrease miR-128-3p expression, thereby resulting in partial abolition of the translational repression of its target gene *GOLM1* in PC cells. Therefore, we hope that the underlying molecular mechanisms of TP73-AS1 could provide a novel therapeutic target for patients with PC.

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## MATERIALS AND METHODS

### Clinical specimens

A total of 116 clinical PC tissues and corresponding normal tissues from surgical resection were collected at Shanghai General Hospital of Shanghai Jiao Tong University between April 2007 and July 2010. PC was diagnosed by pathological examinations. Patients were excluded if they received any treatments such as chemotherapy, radiotherapy, or molecular targeted therapy prior to surgery. All patients provided informed written consent prior to the use of these clinical materials for research purpose. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Shanghai First People's Hospital (No. 2014-07DF), School of Medicine, Shanghai Jiaotong University (Shanghai, China). All human tissues were immediately frozen in liquid nitrogen until being used.

### Cell lines and transfection

Human PC cell lines (SW1990, PANC-1, BXPC-3, AsPc-1, and Capan-1) and human pancreatic duct epithelial cell line (H6C7) were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, United States) supplemented with 10% fatal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Hyclone, South Logan, UT, United States) in a humidified incubator (5% $CO_2$) at 37 °C. Small interfering RNAs (siRNAs) targeting TP73-AS1 (si-TP73-AS1#1, si-TP73-AS1#2, and si-TP73-AS1#3) and negative control (si-control) were purchased from GenePharma (Shanghai, China). MiR-128-3p, miR-NC, anti-miR-128-3p, and anti-miR-NC were obtained from Thermofisher. Cell transfection was performed using FuGENE HD Transfection Reagent (Roche, United States) according to the manufacturer's instructions.

### Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) from PC tissues and cell lines. RNA was reversely transcribed into cDNA using the PrimeScript™RT reagent Kit with gDNA Eraser (TakaRa, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on a Bio-Rad RT-PCR cycler (Bio-Rad, Hercules, United States). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and small RNA RNU6B (U6) were used as the internal controls for lncRNA/mRNA and miRNAs, respectively. Relative expression values of genes were analyzed by the $2^{-\Delta\Delta Ct}$ method.

### Cell proliferation, colony formation, and apoptosis assays

For cell proliferation assay, cells were plated into 96-well plates with four replicate wells per group and then incubated with 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan). The absorbance was measured at 450 nm with a microplate reader (Bio-Tek, Winooski, United States) 2 h later. For colony formation assay, approximately 600 cells were plated into 6-well plates with three replicates. Cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet 14 d later. Cell apoptosis was detected using Annexin-V-fluorescein isothiocyanate apoptosis detection kit (BD, Franklin Lakes, United States) according to the manufacturer's instructions. The apoptosis rate of cells was measured on a BD FACSAria™ II flow cytometer (BD).

### Transwell assay

Cell migration and invasion were evaluated using the Boyden chambers (Millipore; Merck KGaA, Germany) with an 8 mm pore size. Briefly, a total of $2 \times 10^4$ cells in 100 μL serum-free medium were transferred into the upper chamber, and the lower chamber was filled with medium containing 10% FBS. After 24 h incubation, cells were fixed with 10% formaldehyde for 15 min and stained with 0.5% crystal violet for 20 min at room temperate. The migrated cells were counted under an X71 inverted microscope in six randomly selected fields and captured using a microscope (Nikon). The invasion assay was performed in the same way as the migration assay did except that the inserts were pre-coated with Matrigel (BD).

### Luciferase reporter assay

The putative miR-128-3p binding sites in TP73-AS1 and GOLM1 3′UTR were synthesized and inserted into pMIR-REPORT™ miRNA Expression Reporter Vector

(Thermofisher). Their corresponding mutants were generated using MutanBEST Kit (TaKaRa). AsPc-1 and Capan-1 cells were co-transfected with these reporter plasmids and pMIR-REPORT β-gal, miR-128-3p mimics, or miR-128-3p inhibitors using Lipofectamine 3000 (Invitrogen). Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, WI, United States) 48 h after the transfection.

### RNA immunoprecipitation assay

PANC-1 and ASPC-1 cells transfected with miR-NC or miR-128-3p were harvested with revised importance-performance analysis lysis buffer (Cell Signaling Technology, Danvers, MA, United States) containing a proteinase inhibitor cocktail (Roche, IN, United States). The lysates were incubated with magnetic beads conjugated with human anti-Ago2 antibody and normal rabbit immunoglobulin G. Then RNA was isolated from the mixture with TRIzol reagent for qRT-PCR analysis.

### Western blot analysis

Western blot was performed as we previously described[15]. The primary antibodies used in this study are listed as following: Anti-GOLM1 (H00051280-PW1, Abnova, Taiwan), anti-GAPDH (#10494-1-AP, Proteintech, IL, United States), anti-E-cadherin (14-3249-82, CST, United States), anti-N-cadherin (MA1-91128, CST, United States), anti-Vimentin (PA5-27231, CST, United States), anti-Caspase-3 (700182, CST, United States), and anti-Bcl-2 (MA5-11757, CST, United States).

### In vivo xenograft experiment

All animal experiments were performed in compliance to institutional guidelines approved by the Use Committee for Animal Care and this study was approved by the Ethics Committees of Shanghai First People's Hospital of Shanghai Jiao Tong University (approval No. 201804SF). Female BALB/c-nude mice (4–6 wk of age) were purchased from Shanghai SJA Laboratory Animal Company (Shanghai, China) and maintained under specific pathogen free conditions. Capan-1 cells ($1 \times 10^7$; transfected with si-control or si-TP73-AS1#1) mingled with 100 μL serum-free medium were injected subcutaneously into to the flanks of the nude mice. All mice were sacrificed 4 wk after injection and then tumors were isolated and photographed. Tumor volumes were calculated using the formula length × width$^2$/2 and tumor weights were measured. For tail vein injection, $1 \times 10^6$ cells in serum-free medium were injected into 6 wk-old BALB/c-nude mice *via* the tail vein. Five weeks after injection, all mice were sacrificed and lung tissues were finally embedded with paraffin and subjected to hematoxylin and eosin (H&E) staining.

### Statistical analysis

Data are shown as the mean ± SD. The differences between groups were analyzed by Student's *t*-test or Chi-square test. The cumulative overall survival was calculated using the Kaplan-Meier method, and the log-rank test was used to analyze differences in the survival times. Data were analyzed using GraphPad software 7.0. $P < 0.05$ was considered significant.

## RESULTS

### TP73-AS1 is upregulated in PC and associated with a poor prognosis

To explore the roles of TP73-AS1 in PC, we first detected the expression of TP73-AS1 in the human pancreatic duct epithelial cell line (H6C7) and five PC cell lines by qRT-PCR. Our data indicated that TP73-AS1 expression was higher in all PC cell lines than in H6C7, especially in AsPc-1 and Capan-1 cells (Figure 1A). Then the expression of TP73-AS1 in 116 pairs of PC tissues and adjacent non-cancerous tissues was measured, and the results revealed that TP73-AS1 expression was significantly increased in PC tissues compared to the corresponding non-cancerous tissues (Figure 1B and C). Furthermore, it was shown that in the tissue samples of stages I and II PC patients, the expression levels of TP73-AS1 were lower than those in stage III PC patients (Figure 1D). Moreover, the associations between TP73-AS1 expression and the clinicopathological characteristics in PC patients were analyzed. The results suggested that increased TP73-AS1 expression was significantly correlated with tumor size, vessel infiltration, and TNM stage. Besides, no correlation was found between TP73-AS1 expression and other clinical pathological features (Table 1). Kaplan-Meier

**Table 1 Relationship between TP73-AS1 expression and clinicopathological features**

| Characteristic | (*n*) | TP73-AS1 expression | | *P* value |
|---|---|---|---|---|
| | *n* = 116 | Low expression | High expression | |
| Age | | | | 0.7101 |
| < 60 | 65 | 32 | 33 | |
| ≥ 60 | 51 | 23 | 28 | |
| Gender | | | | 0.7026 |
| Male | 74 | 34 | 40 | |
| Female | 42 | 21 | 21 | |
| Tumor differentiation | | | | 0.5723 |
| Poor | 69 | 31 | 38 | |
| Middle and well | 47 | 24 | 23 | |
| Tumor size | | | | 0.008 |
| ≤ 2 cm | 48 | 31 | 17 | |
| > 2 cm | 68 | 26 | 42 | |
| Tumor site | | | | 0.4555 |
| Head | 64 | 28 | 36 | |
| Body | 52 | 27 | 25 | |
| Vessel infiltration | | | | 0.001 |
| Negative | 82 | 47 | 35 | |
| Positive | 34 | 8 | 26 | |
| Lymph node metastasis | | | | 0.5526 |
| No | 78 | 35 | 43 | |
| Yes | 38 | 20 | 18 | |
| TNM stage | | | | 0.0008 |
| I-II | 84 | 48 | 36 | |
| III | 32 | 7 | 25 | |

TNM: Tumor-node-metastasis.

survival results suggested that patients with higher TP73-AS1 expression had a shorter overall survival than those with lower TP73-AS1 expression (Figure 1E). These data indicated that TP73-AS1 might play a vital role in the progression of PC.

### *LncRNA TP73-AS1 is required for efficient PC cell proliferation, migration, and invasion*

In order to assess the biological functions of TP73-AS1, we knocked down TP73-AS1 by transfecting specific siRNAs in AsPc-1 and Capan-1 cells, which have higher endogenous TP73-AS1 expression. The knockdown efficacy was confirmed by qRT-PCR analysis. The expression of TP73-AS1 was markedly decreased in AsPc-1 and Capan-1 cells after transfecting with siRNAs targeting TP73-AS1 (Figure 2A). CCK-8 (Figure 2B) and colony formation assay (Figure 2C) showed that knockdown of TP73-AS1 in PC cells markedly restrained cell proliferation. Furthermore, cell apoptosis was highly promoted by depletion of TP73-AS1 in AsPc-1 and Capan-1 cells (Figure 2D). In addition, in the transwell assay, TP73-AS1 silencing could effectively impede the invasive ability of PC cells (Figure 2E and F). These data revealed that TP73-AS1 acts as an oncogene and depletion of TP73-AS1 inhibits PC cell growth and invasion *in vitro*.

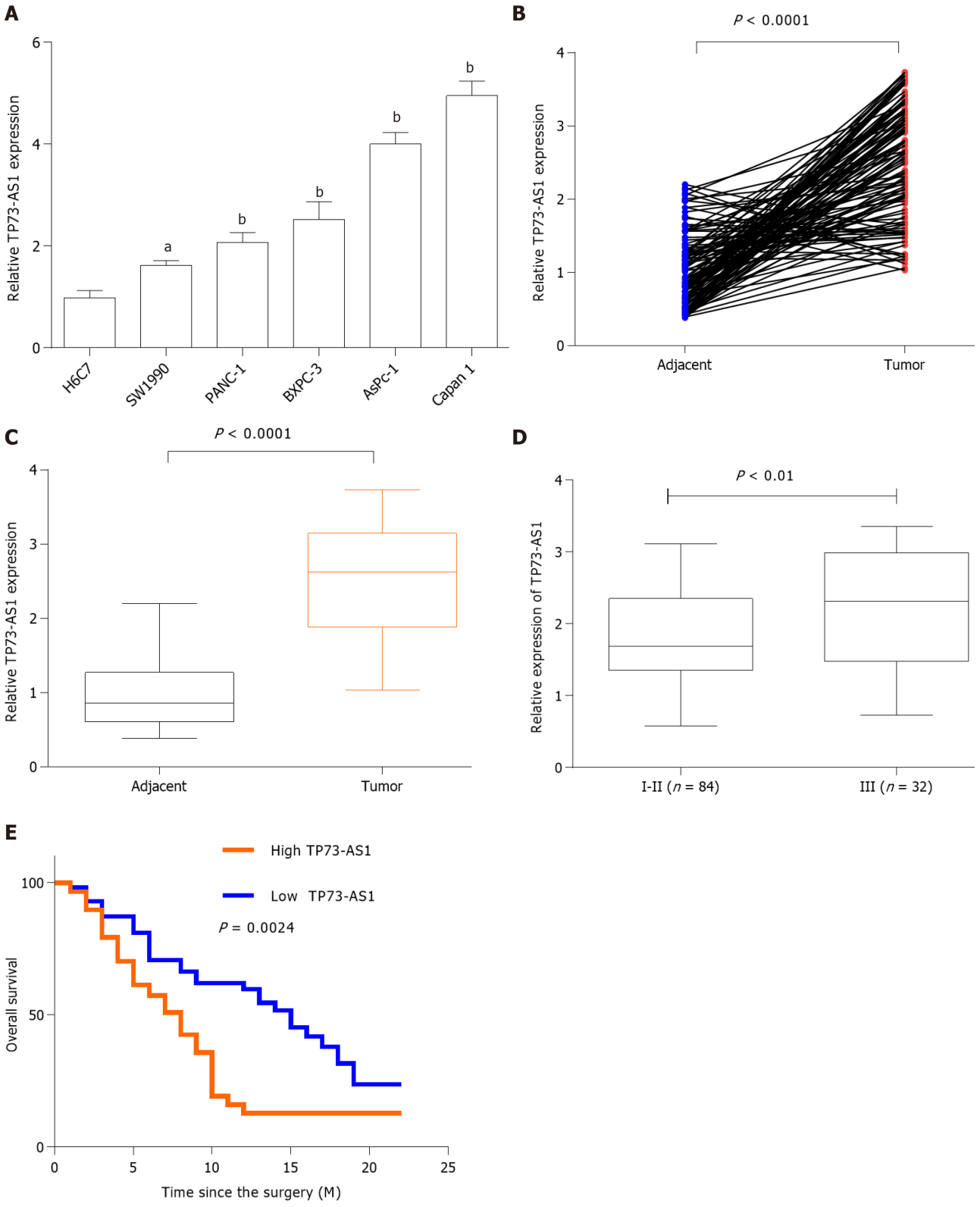
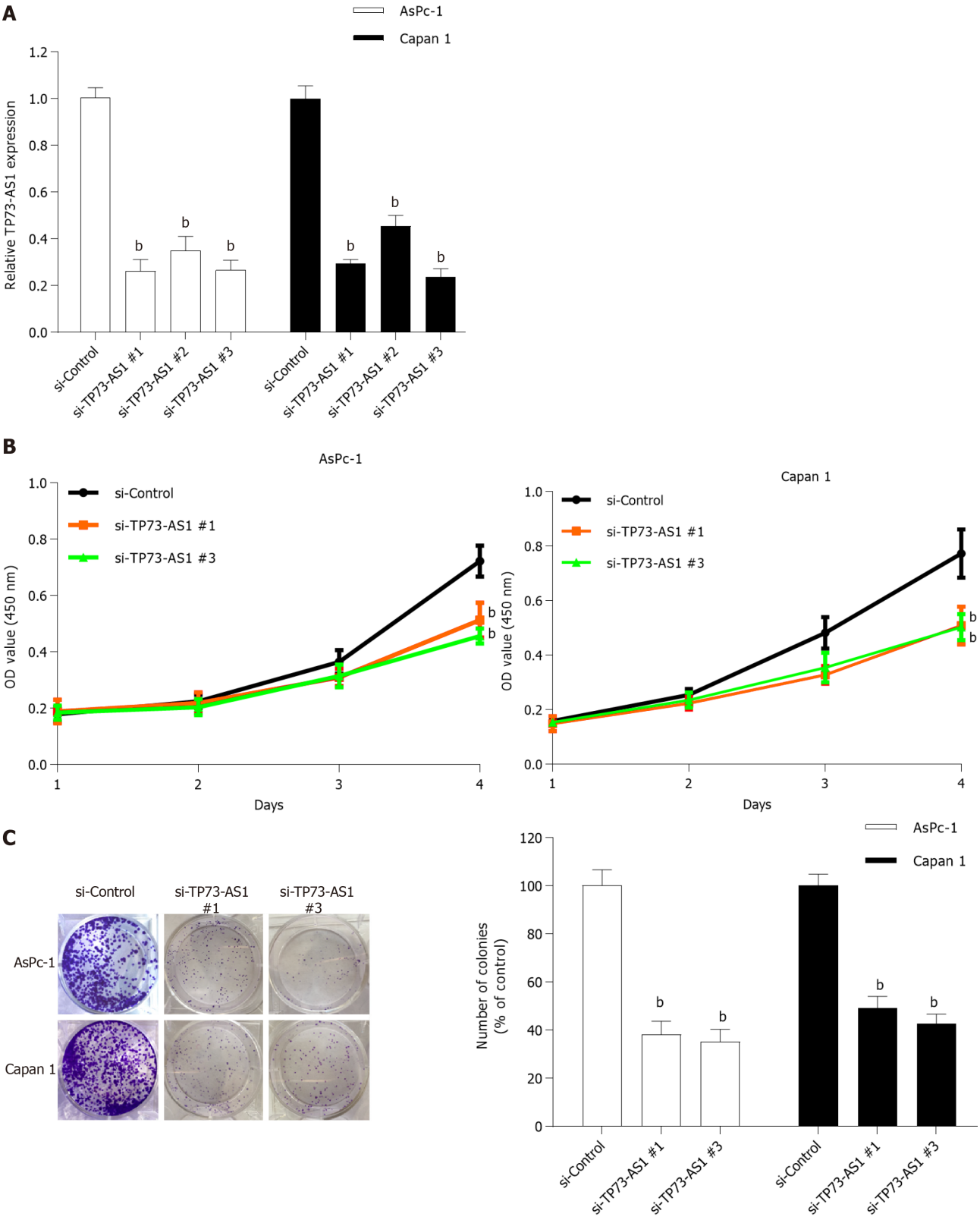



**Figure 1 Aberrantly expressed TP73-AS1 in pancreatic cancer cells and tissues.** A: TP73-AS1 expression in pancreatic cancer (PC) cell lines and an immortalized human pancreatic duct epithelial cell (H6C7) detected by quantitative real-time polymerase chain reaction; B: The relative expression of TP73-AS1 in 116 paired PC tissues and adjacent non-tumor tissues; C: TP73-AS1 is highly expressed in collected PC tissues; D: The different expression of TP73-AS1 between tumor-node-metastasis (TNM) stage I-II; and TNM stage III; E: Kaplan-Meier analysis of the overall survival in patients with PC based on the levels of TP73-AS1 expression. $^{a}P < 0.05$; $^{b}P < 0.01$.

### *LncRNA TP73-AS1 functions as a competing endogenous RNA and sponges miR-128-3p in PC cells*

Accumulating evidence suggests that lncRNAs could bind to miRNAs and function as a molecular sponge in the tumorigenesis of various cancers[4]. To elucidate the


A
AsPc-1
Capan 1
Relative TP73-AS1 expression
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #2
si-TP73-AS1 #3
B
AsPc-1
Capan 1
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
OD value (450 nm)
Days
C
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
AsPc-1
Capan 1
Number of colonies (% of control)

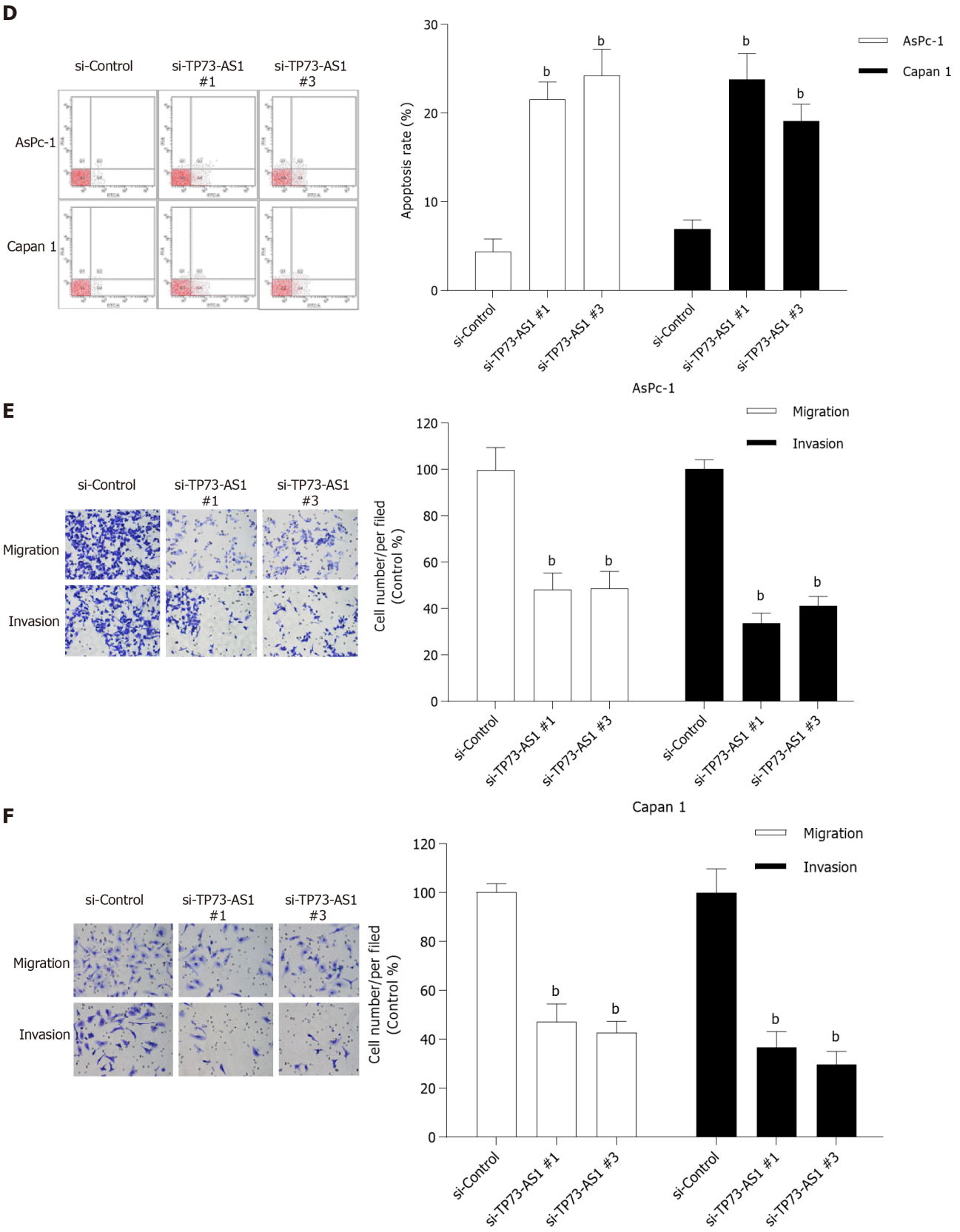



**Figure 2 Knockdown of TP73-AS1 suppresses cell proliferation and invasion *in vitro*.** A: Quantitative real-time polymerase chain reaction analysis of TP73-AS1 expression in AsPc-1 and Capan-1 cells transfected with si-control and small interfering RNAs targeting TP73-AS1; B: CCK-8 proliferation assay in pancreatic cancer cells transfected with si-control, si-TP73-AS1#1, or si-TP73-AS1#3; C: Colony formation assay in AsPc-1 and Capan-1 cells transfected with si-control, si-TP73-AS1#1, or si-TP73-AS1#3; D: Role of TP73-AS1 on AsPc-1 and Capan-1 cells apoptosis checked by flow cytometry assay; E and F: Effect of TP73-AS1 silencing on the migration and invasion ability of AsPc-1 (E) and Capan-1 (F) cell determined by transwell assay. $^{a}P < 0.05$; $^{b}P < 0.01$.

underlying mechanism of TP73-AS1 involved in PC progression, the potential target miRNAs of TP73-AS1 were forecasted with bioinformatics analysis software ( http://starbase.sysu.edu.cn). Among these potential targets, miR-128-3p was chosen for further study because it had been validated as a tumor suppressor in PC (Figure 3A)[16]. Dual-luciferase reporter assay was used to validate the relationship between TP73-AS1 and miR-128-3p. The activity of the wild-type luciferase reporter gene was significantly reduced following transfection with miR-128-3p mimics, whereas the activity of the reporter gene containing the mutant sequence showed no significant change, which indicated that TP73-AS1 could bind to the specific sites of miR-128-3p (Figure 3B). Moreover, anti-Ago2 RNA immunoprecipitation in AsPc-1 and Capan-1 cells transiently overexpressing miR-128-3p could significantly increase the amount of TP73-AS1 (Figure 3C), which could further validate their binding potential. The expression of miR-128-3p was significantly increased in PC cells transfected with si-TP73-AS1#1 and si-TP73-AS1#3 (Figure 3D). Then, we measured miR-128-3p expression level and the relationship between TP73-AS1 and miR-128-3p expression in PC tissues. Interestingly, qRT-PCR assay showed that the miR-128-3p level was remarkably reduced in PC tissues (Figure 3E) and the endogenous miR-128-3p level was negatively correlated with TP73-AS1 in PC tissues (Figure 3F). These results suggested that TP73-AS1 might function as a competing endogenous RNA (ceRNA) for miR-128-3p.

### *MiR-128-3p-3p inhibits pancreatic cell proliferation, migration, and invasion*

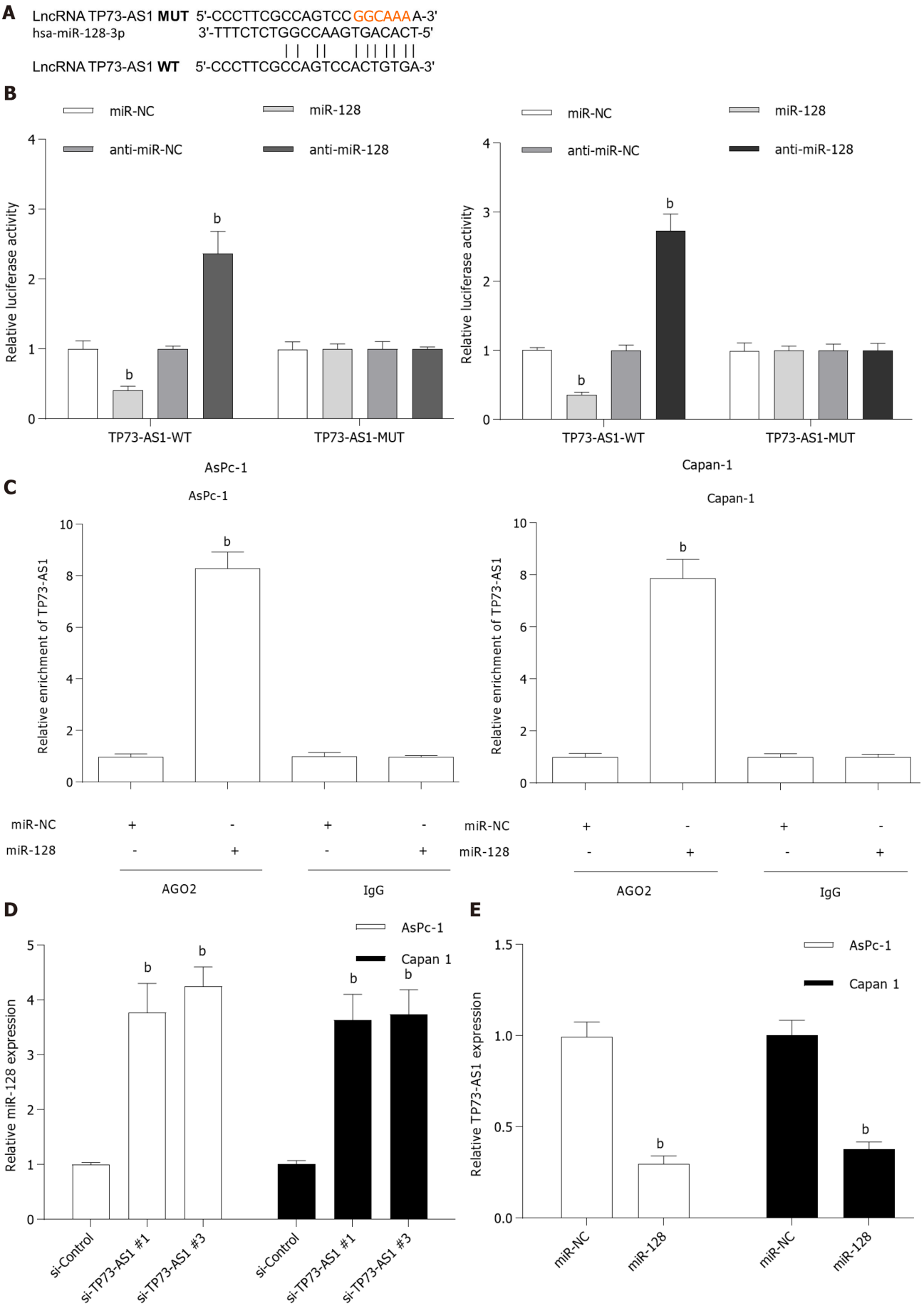
MiR-128-3p was reported to be a tumor suppressor in several cancers, including PC[16,17]. But its effects in the progression of PC are largely unknown. To explore the roles of miR-128-3p in PC cell growth and mobility, miR-128-3p mimics were transfected in AsPc-1 and Capan-1 cells (Figure 4A). CCK-8 (Figure 4B) and colony formation assays (Figure 4C) revealed that miR-128-3p had significant negative regulation effects on the ability of cell proliferation in AsPc-1 and Capan-1 cells. Flow cytometry assay indicated that overexpression of miR-128-3p increased the number of apoptotic cells both in AsPc-1 and Capan-1 cells (Figure 4D). Meanwhile, transwell assays demonstrated that miR-128-3p overexpression significantly restrained PC cell migration and invasion (Figure 4E and F). Considering the downregulation of miR-128-3p in PC tissues, our results manifested that miR-128-3p could act as a tumor suppressor by regulating PC cell proliferation and invasion.

### *Inhibition of miR-128-3p partly reverses regulatory effects induced by depletion of TP73-AS1*

To determine if TP73-AS1 knockdown could exert anti-proliferation and anti-metastasis function by mediating miR-128-3p, anti-miR-128-3p was transfected into AsPc-1 and Capan-1 cells after TP73-AS1 silencing. Functional experiments demonstrated that the TP73-AS1-mediated pro-proliferation (Figure 5A) and anti-apoptosis (Figure 5B) effect was dramatically abrogated by anti-miR-128-3p transfection in TP73-AS1 silencing PC cells. In addition, the inhibitory effects of TP73-AS1 silencing on cell metastasis were rescued by anti-miR-128-3p transfection (Figure 5C and D). Moreover, epithelial-mesenchymal transition (EMT)-related proteins were detected by Western blot. Consistent with the functional assays above, the results showed that TP73-AS1 could regulate EMT-related proteins by regulating miR-128-3p (Figure 5E). All these data indicated that TP73-AS1 is involved in PC progression, at least partly through miR-128-3p.

### *LncRNA TP73-AS1 regulates GOLM1 expression by competing for miR-128-3p-3p*

Since TP73-AS1 was demonstrated to bind to miR-128-3p, we assessed whether TP73-AS1 could indirectly affect the target gene of miR-128-3p by serving as a ceRNA. Based on online bioinformatics analysis, GOLM1 3'UTR was found to possess a putative recognition site for miR-128-3p (Figure 6A). The luciferase reporter assay was carried out and the results showed that the luciferase activity of plasmid carrying GOLM1 3'UTR-WT was significantly decreased by transfecting miR-128-3p mimics both in AsPc-1 and Capan-1 cells (Figure 6B). However, these effects were abolished when the binding sequences were mutated. Transfecting miR-128-3p mimics led to a significant decrease of GOLM1 mRNA and protein expression in AsPc-1 and Capan-1 cells (Figure 6C). To further explore the correlation between TP73-AS1 and GOLM1, the mRNA and protein expression of GOLM1 was detected after TP73-AS1 silencing. As expected, the mRNA and protein expression of GOLM1 was remarkably decreased after silencing TP73-AS1 both in AsPc-1 and Capan-1 cells (Figure 6D). Moreover, the level of GOLM1 mRNA was significantly down-regulated in PC tissues compared to


A
LncRNA TP73-AS1 MUT 5'-CCCTTCGCCAGTCC GGCAAA A-3'
hsa-miR-128-3p 3'-TTTCTCTGGCCAAGTGACACT-5'
LncRNA TP73-AS1 WT 5'-CCCTTCGCCAGTCCACTGTGA-3'
B
miR-NC
miR-128
anti-miR-NC
anti-miR-128
Relative luciferase activity
TP73-AS1-WT
TP73-AS1-MUT
AsPc-1
Capan-1
C
Relative enrichment of TP73-AS1
miR-NC
miR-128
AGO2
IgG
D
Relative miR-128 expression
si-Control
si-TP73-AS1 #1
si-TP73-AS1 #3
E
Relative TP73-AS1 expression

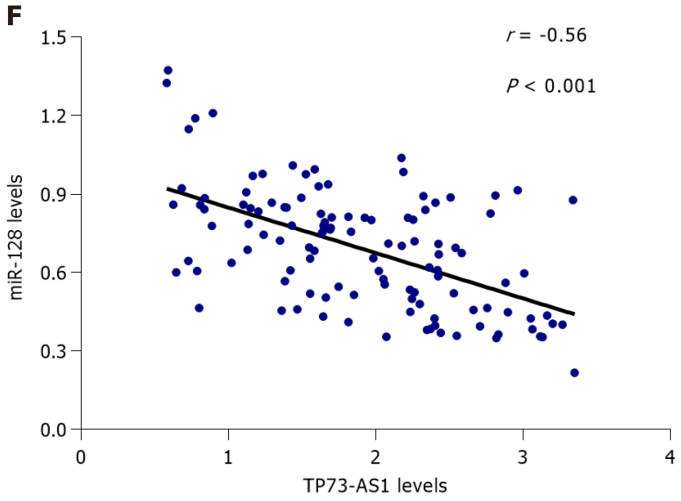



**Figure 3 TP73-AS1 physically interacts with miR-128-3p-3p.** A: miR-128-3p and its predicted binding sites in the TP73-AS1 sequence; B: Luciferase activity of pancreatic cancer (PC) cells co-transfected with miRNA mimics and luciferase reporter vectors containing TP73-AS1-WT or TP73-AS1-MUT; C: RNA immunoprecipitation assay of the enrichment of TP73-AS1 in AsPc-1 and Capan-1 transfected with miR-NC or miR-128-3p. Immunoglobulin G was used as negative control; D: Expression levels of miR-128-3p in AsPc-1 and Capan-1 cells transfected with si-control or small interfering RNAs targeting TP73-AS1; E: Expression levels of miR-128-3p in PC tissues and adjacent non-tumor tissues; F: The expression of TP73-AS1 and miR-128-3p exhibits a negative correlation in PC tissues. $^{b}P < 0.01$. IgG: Immunoglobulin G.

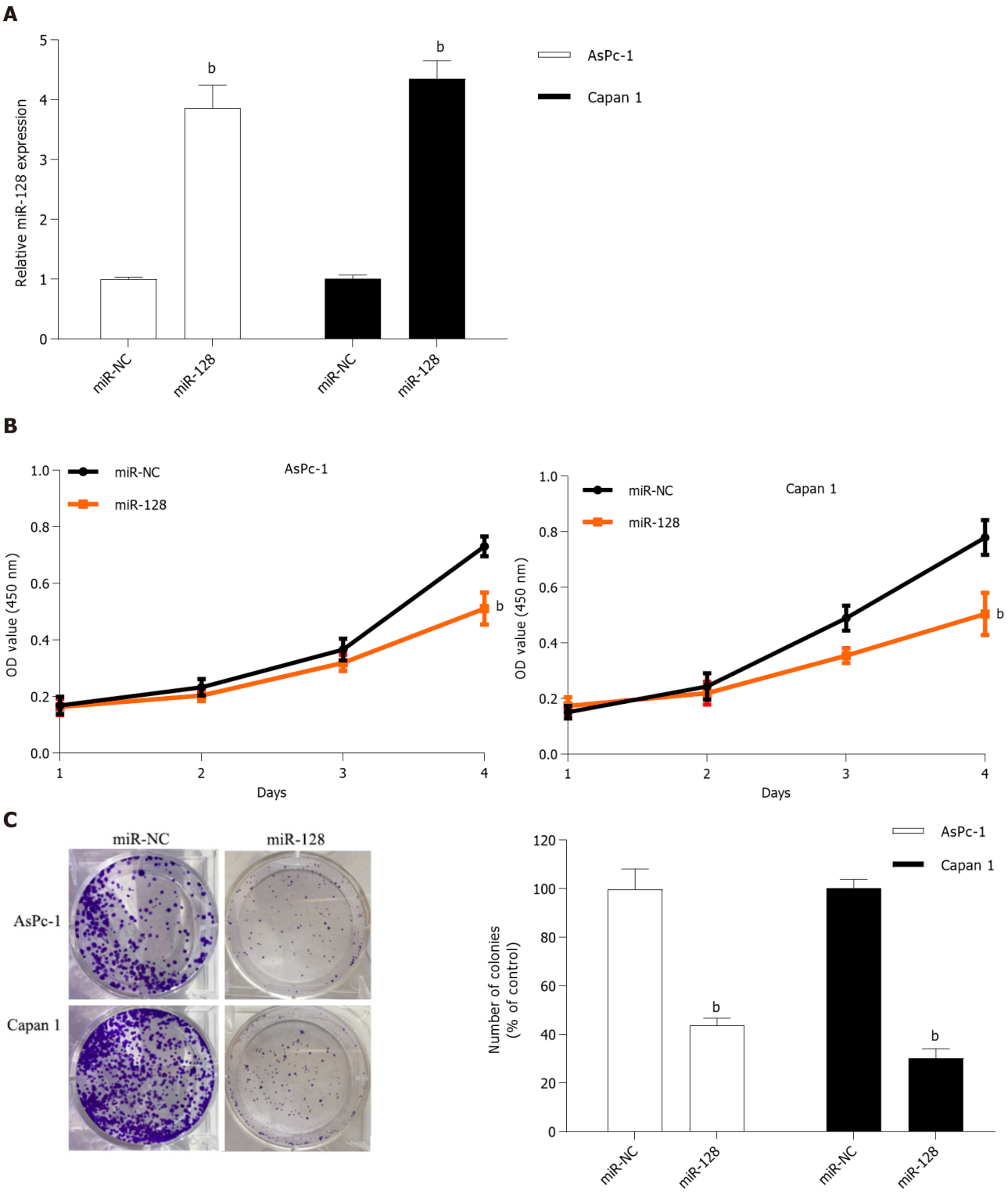
the corresponding non-cancerous tissues (Figure 6E). Interestingly, an inverse correlation was identified between miR-128-3p and GOLM1 mRNA levels in PC tissues (Figure 6F). In contrast, the positive relationship between TP73-AS1 and GOLM1 mRNA levels was observed in PC tissues (Figure 6G). The protein expression of GOLM1 was increased after anti-miR-128-3p transfection in TP73-AS1 silencing PC cells (Figure 6H), which suggested that TP73-AS1 could regulate the expression of GOLM1 by acting as a sponge for miR-128-3p *in vitro*.

### *TP73-AS1 silencing inhibits tumor growth and metastasis of PC cells*

To further elucidate the biological roles of TP73-AS1 in PC tumorigenesis *in vivo*, Capan-1 cells transfected with si-TP73-AS1#1 or si-Control were implanted into nude mice *via* subcutaneous injection. Four weeks later, the subcutaneous tumors were collected. Tumor growth curve and tumor weight from the si-TP73-AS1#1 group showed lower size and lighter tumor weight (Figure 7A-C). Moreover, qRT-PCR analysis suggested that the expression of TP73-AS1 was decreased (Figure 7D) and the expression of miR-128-3p (Figure 7E) was increased in the si-TP73-AS1#1 group. In addition, Ki-67 immunostaining indicated that the subcutaneous tumors formed by TP73-AS1 silencing Capan-1 cells showed fewer Ki-67 positive cells compared to the control group (Figure 7F). Together, the *in vitro* and *in vivo* results suggested that TP73-AS1 might function as an oncogene in the progression of PC. To investigate the metastatic potential of TP73-AS1 *in vivo*, Capan-1 cells transfected with si-TP73-AS1#1 or si-Control were injected into the mice *via* the tail vein. As shown in Figure 7G, silencing TP73-AS1 remarkably decreased the number and size of lung metastatic lesions as detected by H&E staining. Moreover, we detected apoptotic markers in the tumors from the two groups and the results showed that apoptosis-related genes were significantly altered (Figure 7H).

## DISCUSSION

Increasing numbers of studies have shown that lncRNAs are involved in both normal development and pathological processes of human diseases by chromatin modification, genomic imprinting, RNA decay, and sponge-like miRNAs[5,18,19]. Dysregulation of lncRNAs might influence cell proliferation, metastasis, angiogenesis, and drug resistance[18,20,21]. It has been previously reported that increased expression of TP73-AS1 is associated with a poorer prognosis and shorter survival in patients with hepatocellular carcinoma[13]. High TP73-AS1 expression was also observed and associated with poor overall survival of patients with osteosarcoma[22]. TP73-AS1 was up-regulated in both colorectal cancer tissues and colorectal cancer cells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Relative miR-128 expression
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Capan 1
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miR-NC
miR-128
B
AsPc-1
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miR-NC
miR-128
AsPc-1
Capan 1
Number of colonies (% of control)

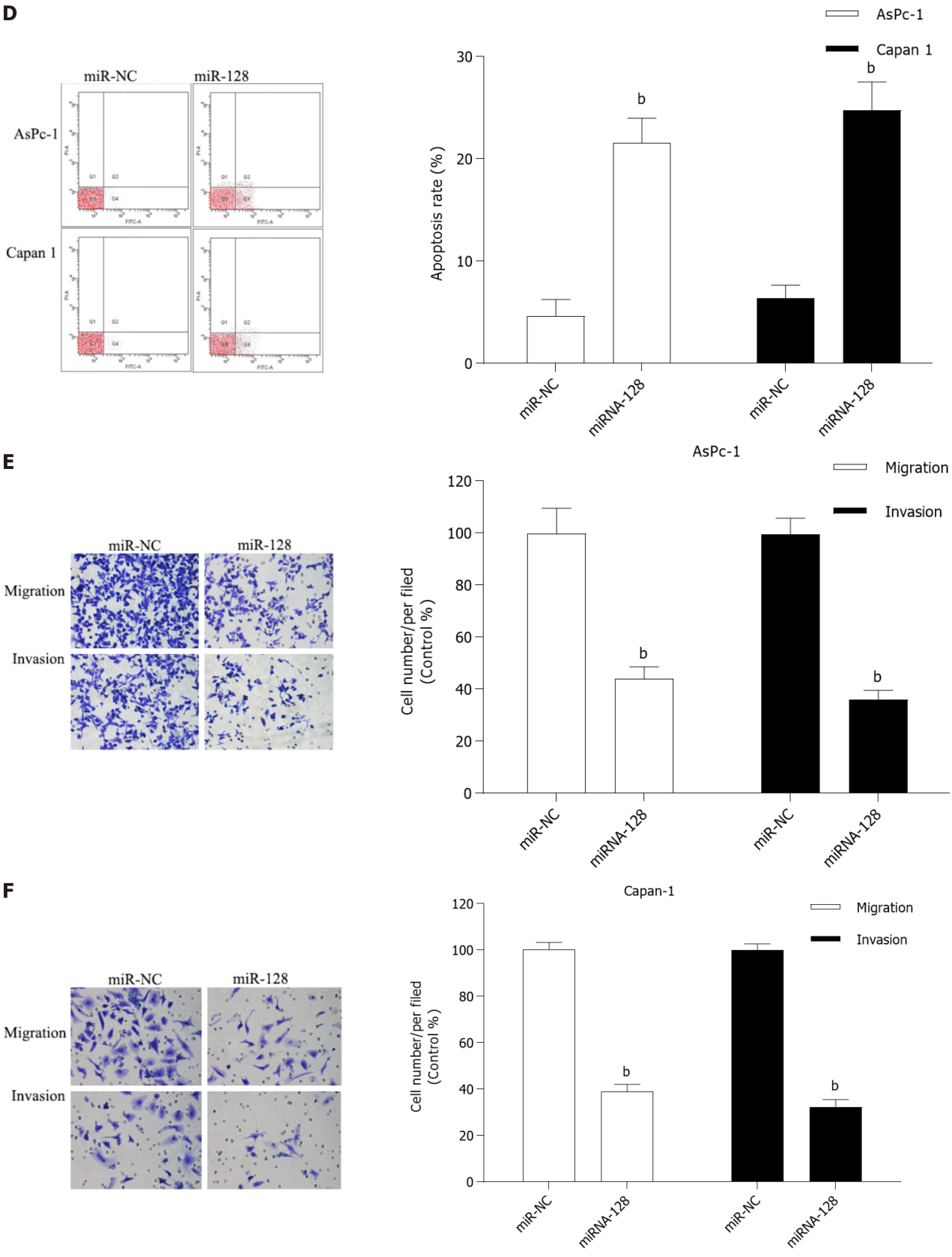



**Figure 4 MiR-128-3p inhibits the proliferation, migration, and invasion of pancreatic cancer cells.** A: The efficiencies of overexpression for miR-128-3p determined by quantitative real-time polymerase chain reaction; B and C: CCK-8 assay (B) and colony formation assay (C) for detecting cell proliferative ability after overexpression of miR-128-3p; D: Effect of miR-128-3p on pancreatic cancer cell apoptosis determined by flow cytometry; E and F: Transwell assay with or without matrigel for assessing the effect of miR-128-3p on cell migration and invasion in AsPc-1 (E) and Capan-1 (F) cells. $^{b}P < 0.01$.

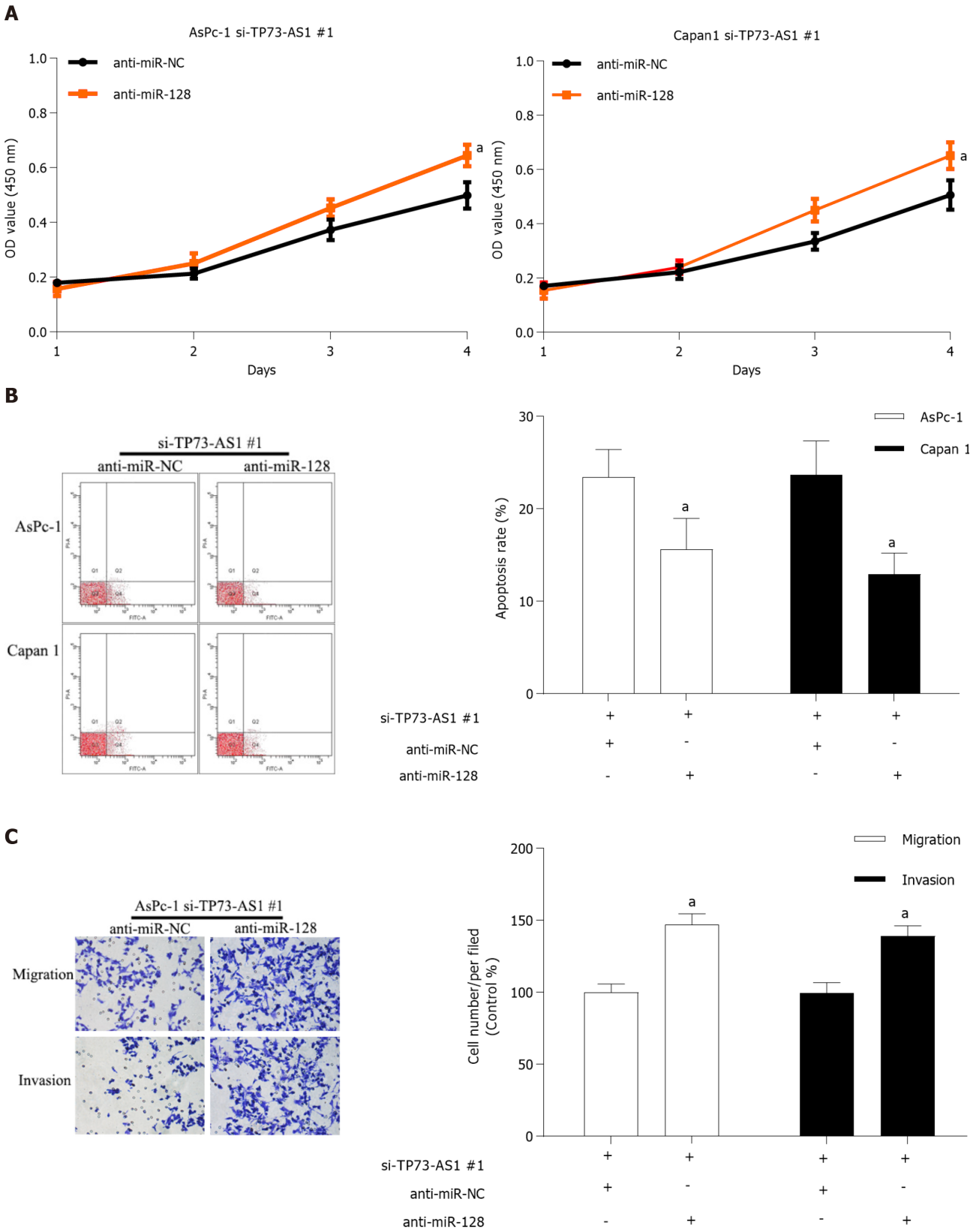
and high TP73-AS1 expression was associated with metastasis and advanced clinical stages in patients with colorectal cancer[23]. Above studies suggested that TP73-AS1 might act as an oncogene in tumor progression, which encouraged us to explore the expression and biological function of TP73-AS1 in PC. In agreement with these studies, we found that TP73-AS1 was significantly increased and associated with tumor size, vessel infiltration, and poor prognosis in PC patients. Furthermore, our results showed that knockdown of TP73-AS1 suppressed the proliferation and invasion of PC cells *in vitro* and the tumor growth *in vivo*.

Emerging studies demonstrated that lncRNAs could function as ceRNAs to regulate gene expression through competitively binding to miRNAs[4,5]. To further investigate the mechanism of the TP73-AS1 in PC, bioinformatics analysis predicted that TP73-AS1 is a target of miR-128-3p. Numerous studies have indicated that miR-128-3p could act as a tumor suppressive role in many tumors, including glioma, breast cancer, and non-small cell lung cancer[24-26]. In our present study, we found that miR-128-3p could significantly suppress PC cell growth and invasion. Luciferase reporter assay confirmed the relationship between TP73-AS1 and miR-128-3p. Mechanistical study showed that TP73-AS1 could mediate PC cell proliferation, migration, and invasion by sponging miR-128-3p and a negative correlation between TP73-AS1 and miR-128-3p expression was observed in PC tissues. Further investigation indicated that the 3'UTR of *GOLM1* harbors a functional response element for miR-128-3p. GOLM1, a type II transmembrane protein, has been reported to be induced by virus infection[27,28]. Recent studies have shown that GOLM1 commonly expressed in epithelial cells of normal tissues was significantly upregulated in tumor tissues, which suggested a possible oncogenic role of GOLM1 in tumor progression[29,30]. Moreover, clinicopathological features showed that GOLM1 was correlated with Edmondson grade, vascular invasion, TNM stage, overall survival, as well as Vimentin expression[31]. GOLM1 was also reported to promote prostate cancer cell growth, migration, and invasion, and inhibited cell apoptosis *via* the PI3K/AKT/mTOR signaling axis[32]. The role of GOLM1 was unclear in PC and our data showed that the mRNA expression of *GOLM1* was increased in PC tissues. Moreover, ectopic expression of miR-128-3p significantly inhibited the expression of GOLM1 at both the mRNA and protein level. Most interestingly, miR-128-3p-3p could abrogate TP73-AS1-mediated expression of GOLM1, which suggested that TP73-AS1 could act as a molecular sponge to decrease miR-128-3p expression, thereby resulting in partial abolition of the translational repression of its target gene *GOLM1* in PC cells.

*KRAS* gene, the most common genetic driver in PC, is mutated in about 93% of PC s[33,34]. The KRAS protein is a small GTPase, which is responsible for interacting with cell membrane growth factor receptors and controlling the switch of multiple signaling pathways and cellular processes. Oncogenic KRAS mutations have been found in 95% of pancreatic ductal adenocarcinoma tissues[35,36]. Decades of research have discovered and clarified the complex picture of KRAS-regulated biological processes, including cell metabolism, tumor cell signaling, the tumor microenvironment, micropinocytosis, apoptosis, and redox homeostasis[37,38]. In our research, ASPC-1 and Capan-1 cells were the two PC cell lines that we selected, both of which contained mutations in the *KRAS* gene. As our results show, the regulatory roles of TP73-AS1 in cell proliferation, migration, and invasion ability were verified by Cell Counting Kit-8, wound-healing, and transwell assays in ASPC-1 and Capan-1 cells. Due to the vital role that KRAS could play in PC, we are also curious about the role of TP73-AS1 in KRAS wild cells. Therefore, in our further research, we would select BXPC-3 cell line, which contains wild *KRAS* gene, for *in vitro* and *in vivo* functional assays of TP73-AS1 to detect whether *KRAS* gene could modulate the function of TP73-AS1 in PC.

## CONCLUSION

In summary, our data suggested that TP73-AS1 could function as an oncogenic lncRNA in PC progression. Moreover, TP73-AS1 could promote tumor growth and invasion by acting as a ceRNA to promote GOLM1 expression by sponging miR-128-3p in PC.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anti-miR-128
OD value (450 nm)
Days
a
Capan1 si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
OD value (450 nm)
Days
a
B
si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
AsPc-1
Capan 1
AsPc-1
Capan 1
Apoptosis rate (%)
a
a
si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
C
AsPc-1 si-TP73-AS1 #1
anti-miR-NC
anti-miR-128
Migration
Invasion
Migration
Invasion
Cell number/per filed (Control %)
a
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anti-miR-128

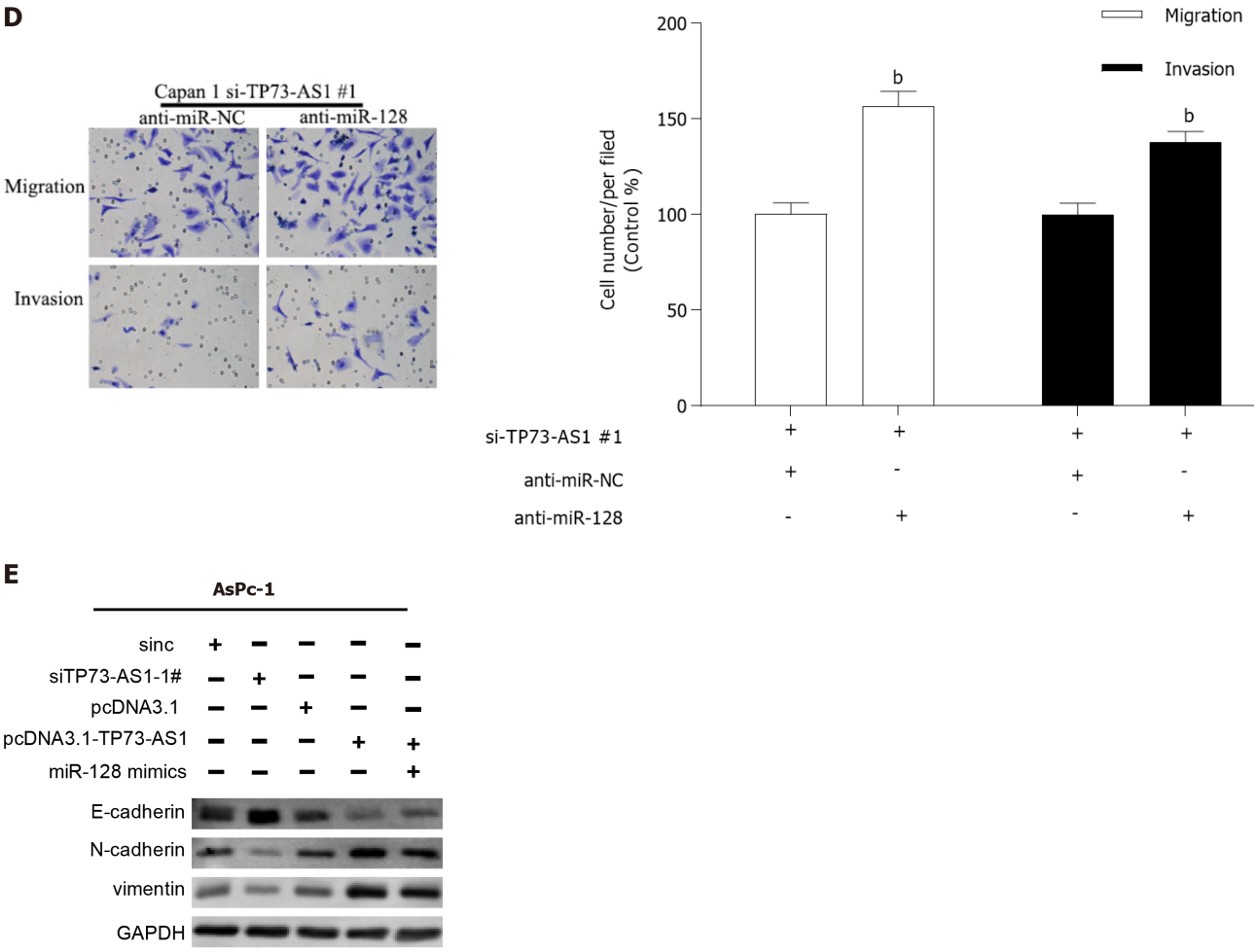
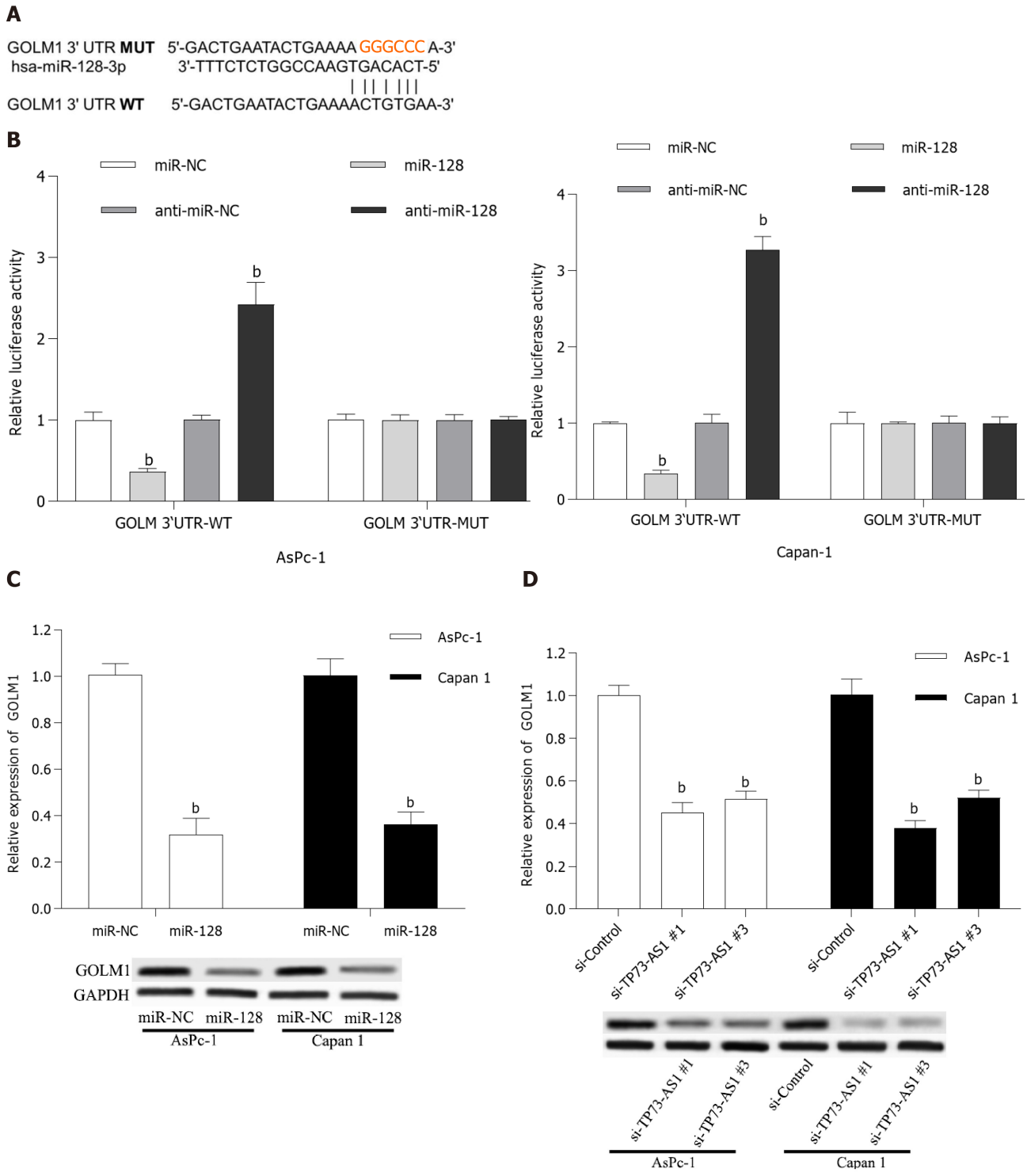



**Figure 5 MiR-128-3p is responsible for TP73-AS1-mediated malignant behavior.** A: Knockdown of miR-128-3p eliminates the inhibition on cell proliferation by TP73-AS1 depletion; B: Depletion of miR-128-3p abolishes cell apoptosis induced by TP73-AS1 silencing; C and D: Representative images of migrated and invaded cells treated with si-TP73-AS1#1 and anti-miR-NC or anti-miR-128-3p in AsPc-1 (C) and Capan-1 (D) cells; E: Epithelial-mesenchymal transition-related proteins detected by Western blot. $^{a}P < 0.05$; $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.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hsa-miR-128-3p 3'-TTTCTCTGGCCAAGTGACACT-5'
GOLM1 3' UTR WT 5'-GACTGAATACTGAAAACTGTGAA-3'
B
miR-NC
miR-128
anti-miR-NC
anti-miR-128
Relative luciferase activity
GOLM 3'UTR-WT
GOLM 3'UTR-MUT
AsPc-1
Capan-1
C
AsPc-1
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GAPDH
D
si-Control
si-TP73-AS1 #1
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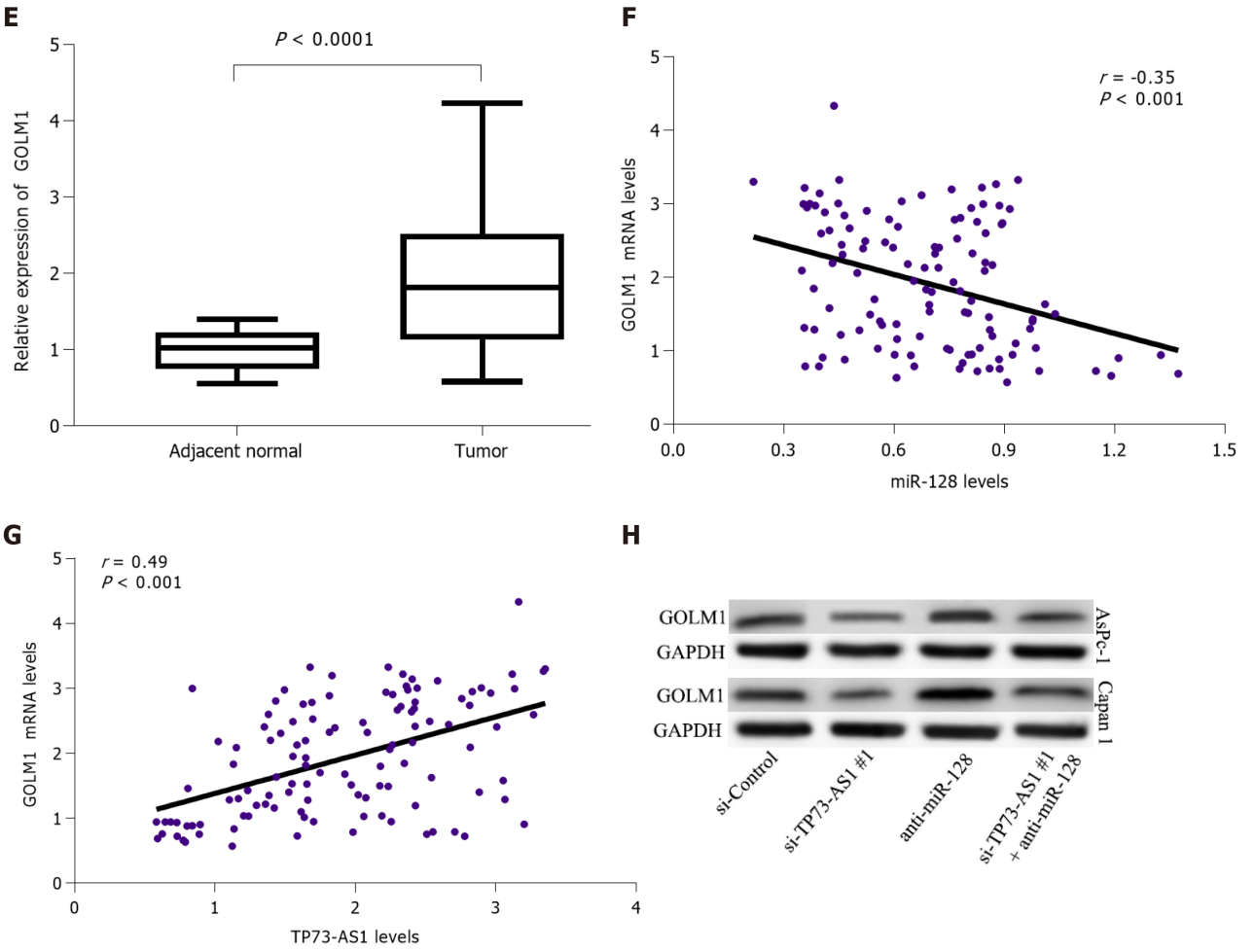
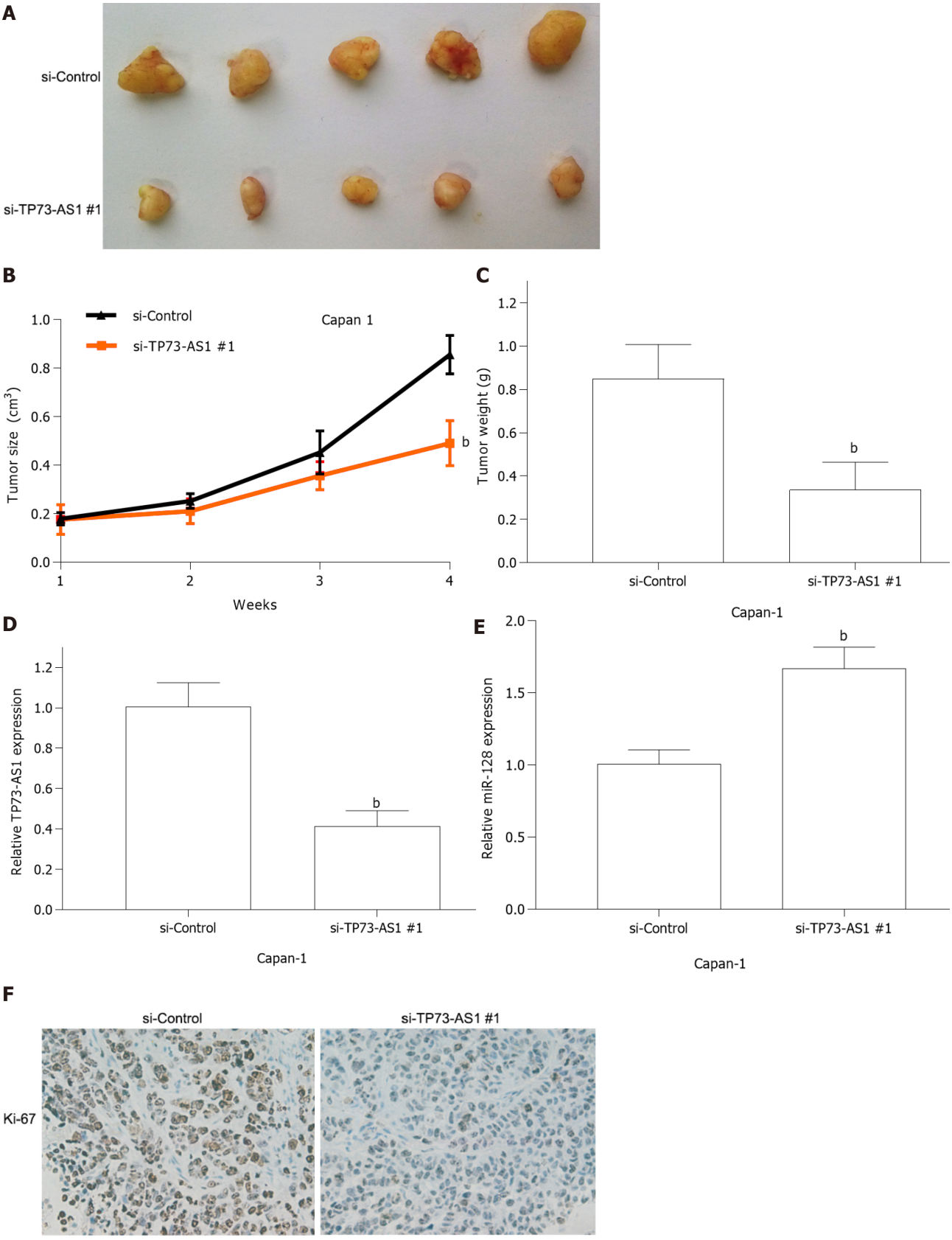



**Figure 6 TP73-AS1 increases GOLM1 expression by decreasing miR-128-3p.** A: Schematic diagram presenting the putative miR-128-3p recognition site in the GOLM1 sequence; B: Luciferase activity in pancreatic cancer (PC) cells transfected with miR-128-3p and reporter plasmids containing WT or MUT GOLM1 3'UTR; C: The GOLM1 mRNA (up) and protein (down) levels in AsPc-1 and Capan-1 cells with miR-128-3p transfection; D: The GOLM1 mRNA (up) and protein (down) levels in AsPc-1 and Capan-1 cells after TP73-AS1 silencing; E: The expression levels of *GOLM1* mRNA in 116 pairs of PC and adjacent non-tumor tissues assessed by quantitative real-time polymerase chain reaction; F: The relationship between miR-128-3p and GOLM1 mRNA levels assessed by Pearson correlation analysis; G: The relationship between TP73-AS1 and GOLM1 mRNA levels assessed by Pearson correlation analysis; H: The protein expression levels of GOLM1 detected by Western blot assay as indicated. $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.


A
si-Control
si-TP73-AS1 #1
B
si-Control
si-TP73-AS1 #1
Capan 1
Tumor size (cm3)
Weeks
b
C
Tumor weight (g)
b
si-Control
si-TP73-AS1 #1
Capan-1
D
Relative TP73-AS1 expression
b
si-Control
si-TP73-AS1 #1
Capan-1
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Ki-67

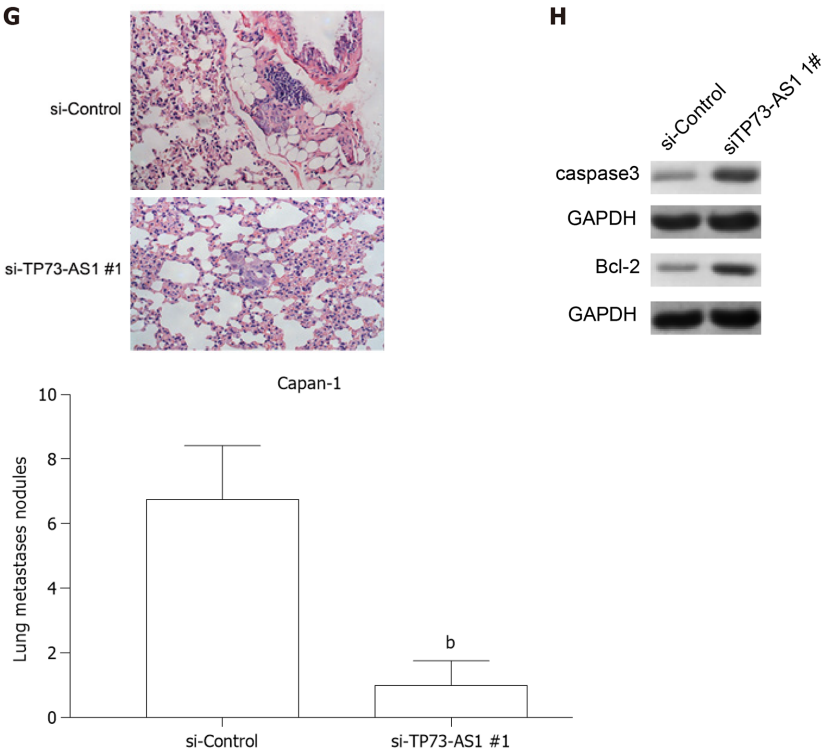


**Figure 7 TP73-AS1 knockdown inhibits tumor growth and metastasis in nude mice.** A: Images of resected tumors from nude mice from the indicated groups. *n* = 5 mice per group; B: Tumor growth curves established by measuring tumor volume after injection at the indicated time points; C: Weights of resected tumors from nude mice from the indicated groups; D and E: Quantitative real-time polymerase chain reaction to detect the expression of TP73-AS1 (D) and miR-128-3p (E) in xenograft tumor tissues; F: Ki67 expression in resected tumor tissues evaluated by immunohistochemistry analysis; G: Representative images and quantification of hematoxylin and eosin staining of lungs isolated from mice; H: Apoptosis markers detected by Western blot. $^{b}P < 0.01$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

## ARTICLE HIGHLIGHTS

### *Research background*

Pancreatic cancer (PC) is the fourth most frequent cause of cancer-related deaths in the world. Emerging evidence has revealed that long non-coding RNAs (lncRNAs) could play crucial roles in the progression of PC. However, the biological role and clinical significance of TP73-AS1 in PC remain unclear.

### *Research motivation*

Treatments for PC are still limited, and surgical resection could be the only chance to obtain curative treatment. We hope to provide a novel therapeutic target for patients with PC.

### *Research objectives*

The present study aimed to investigate the role of TP73-AS1 in the growth and metastasis of PC.

### *Research methods*

Quantitative reverse transcription-polymerase chain reaction was used to detect the expression of lncRNA TP73-AS1, miR-128-3p, and *GOLM1* in PC tissues and cells. The regulatory roles of TP73-AS1 in cell proliferation, migration, and invasion ability were verified by Cell Counting Kit-8, wound-healing, and transwell assays. The bioinformatics prediction software ENCORI was used to predict the putative binding sites of miR-128-3p. The interactions among TP73-AS1, miR-128-3p, and GOLM1 were explored by bioinformatics prediction, luciferase assay, and Western blot.

### *Research results*

Our data suggested that TP73-AS1 and miRNA-128-3p were dysregulated in PC tissues and cells. TP73-AS1 silencing inhibited PC cell proliferation, migration, and invasion *in vitro* as well as suppressed tumor growth *in vivo*. Moreover, TP73-AS1

could promote tumor growth and invasion by acting as a competing endogenous RNA to promote GOLM1 expression by sponging miR-128-3p in PC.

### Research conclusions

TP73-AS1 could promote PC cell proliferation and metastasis by modulating the miR-128-3p/GOLM1 axis.

### Research perspectives

TP73-AS1 could promote PC progression, which might provide a potential treatment strategy for patients with PC.

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ORIGINAL ARTICLE

Retrospective Study

# Prediction of microvascular invasion in solitary hepatocellular carcinoma ≤ 5 cm based on computed tomography radiomics

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## Abstract

### *BACKGROUND*

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide. Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with hepatocellular carcinoma (HCC). Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction.

### *AIM*

To investigate the predictive value of computed tomography radiomics for MVI in solitary HCC ≤ 5 cm.

### *METHODS*

A total of 185 HCC patients, including 122 MVI negative and 63 MVI positive patients, were retrospectively analyzed. All patients were randomly assigned to

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the training group ($n$ = 124) and validation group ($n$ = 61). A total of 1351 radiomic features were extracted based on three-dimensional images. The diagnostic performance of the radiomics model was verified in the validation group, and the Delong test was applied to compare the radiomics and MVI-related imaging features (two-trait predictor of venous invasion and radiogenomic invasion).

*RESULTS*

A total of ten radiomics features were finally obtained after screening 1531 features. According to the weighting coefficient that corresponded to the features, the radiomics score (RS) calculation formula was obtained, and the RS score of each patient was calculated. The radiomics model exhibited a better correction and identification ability in the training and validation groups [area under the curve: 0.72 (95% confidence interval: 0.58-0.86) and 0.74 (95% confidence interval: 0.66-0.83), respectively]. Its prediction performance was significantly higher than that of the image features ($P < 0.05$).

*CONCLUSION*

Computed tomography radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm, and the predictive ability is higher than that of image features.

**Key Words:** Hepatocellular carcinoma; Microvascular invasion; Radiomics; Image features; Computed tomography



**Core Tip:** Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with hepatocellular carcinoma (HCC). This study showed that radiomics as an emerging method at present had a good diagnostic efficiency and exhibited better accuracy in predicting MVI than image features, indicating that radiomics is a more suitable method in predicting MVI in solitary HCC ≤ 5 cm.

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## INTRODUCTION

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide[1]. Furthermore, more than half of liver cancers occur in China, where there is a high incidence of hepatitis B[2]. Moreover, the recurrence rate after hepatectomy is 70%[3], and microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer[4]. MVI has a relatively high incidence in hepatocellular carcinoma (HCC), which ranges from 12.4% to 57.1%, and may occur even in solitary HCC ≤ 2 cm[5,6]. Thus, accurately identifying MVI before surgery is of great importance in making treatment strategies and predicting the prognosis of patients with HCC[7]. However, MVI can only be confirmed by histopathology *via* surgical resection at present. Therefore, the accurate prediction of MVI before surgery is desperately needed. Radiomics as an emerging field, which aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction[8]. At present, few studies have focused on the prediction of MVI in the early stage of HCC (which refers to solitary tumor with a size of ≤ 5 cm, without intrahepatic venous invasion[9]). The present study aimed to

investigate the predictive value of computed tomography (CT) radiomics for MVI in solitary HCC ≤ 5 cm.

## MATERIALS AND METHODS

### Patients

Patients were retrospectively collected from January 1, 2014 to November 15, 2018 (Hunan provincial People's Hospital). The inclusion criteria were: (1) Pathologically diagnosed hepatocellular carcinoma with MVI; (2) Solitary tumor with the maximum diameter of ≤ 5 cm; and (3) Enhanced CT scanning was performed before surgery. The exclusion criteria were: (1) Complicated with other malignant tumors, and multiple primary or recurrent liver cancer; (2) History of preoperative treatment; (3) CT revealed a vascular tumor thrombus or macrovascular invasion; or (4) The tumor boundary was difficult to determine. The flowchart for the screening of patients is presented in Figure 1.

### Examination methods

The Philips (Brilliance iCT 256) and Neusoft (NeuViz 64EN) scanners were used, with a tube voltage of 100-200 kV, tube current of 171-313 mAs, scanning layer thickness of 5 mm, layer spacing of 5 mm, and matrix of 1024 × 1024. The contrast medium (iopromide injection, 300 mgI/mL) was injected using a high-pressure syringe through the anterior cubital vein at a rate of 3.5 mL/s and at a dose of 1.2 mL/kg. Dynamic contrast-enhanced imaging data acquisition was performed at fixed time points: For the arterial phase, acquisition occurred at approximately 25-33 s after administration; for the portal vein phase, it was 57-63 s, and for the delayed phase, 117-123 s.

### Observation of imaging features

The two-trait predictor of venous invasion (TTPVI) was defined as having two independent imaging characteristics at the same time, and the development of an internal tumor artery without the signs of low density at the tumor margin[10]. Radiogenomic invasion (RVI) comprised of three independent image features: Intratumoral artery, low-density ring, and tumor-liver difference[11]. If there was an intratumoral artery, but there was no low-density ring or tumor-liver difference, the tumor was considered to have RVI. The imaging features (TTPVI and RVI) were evaluated double-blindly by two radiologists (with three years and seven years of experience in abdominal radiology, respectively). If these radiologists had inconsistent evaluation results, a third senior radiologist (with 13 years of experience in abdominal radiology) would make the further confirmation. The detailed description is presented in Figure 2.

### Drawing, image feature extraction, selection, and construction of region of interest

**Region of interest selection:** The CT images of the patients in the arterial phase were exported in DICOM format. Without knowing the pathological results, the radiologist with three years of experience in abdominal radiology used the 3D-Slice software ( www.slice.org) to delineate the region of interest (ROI) in each layer that contained the tumor, and finally formed the three-dimensional segmented image.

**Extraction of radiomic features:** After segmenting the images, the plug-in radiologics in the 3D-Slice software was used to analyze the original image data in the ROI, and 1351 candidate texture parameters were extracted, including the histogram features, morphological features, original features, and texture features.

**Selection of features correlated to the MVI status and construction of the radiomics tags:** First, the extracted intraclass features were evaluated by intraclass correlation coefficient (ICC), and features with ICC < 0.75 were removed. Then, the least absolute shrinkage and selection operator (LASSO) algorithm was used for dimension reduction and feature construction, and the least feature variables were selected by 10-fold cross-validation with the minimal value. The selected features were modeled by Logistic regression, in order to generate the formula of the radiomics score (rad-score, RS) and calculate the RS score of each patient.

### Statistical analysis

The R language (version 3.4.0, https://www.r-project.org) was used for the statistical analyses, and $P < 0.05$ was considered statistically significant. The chi-square test for

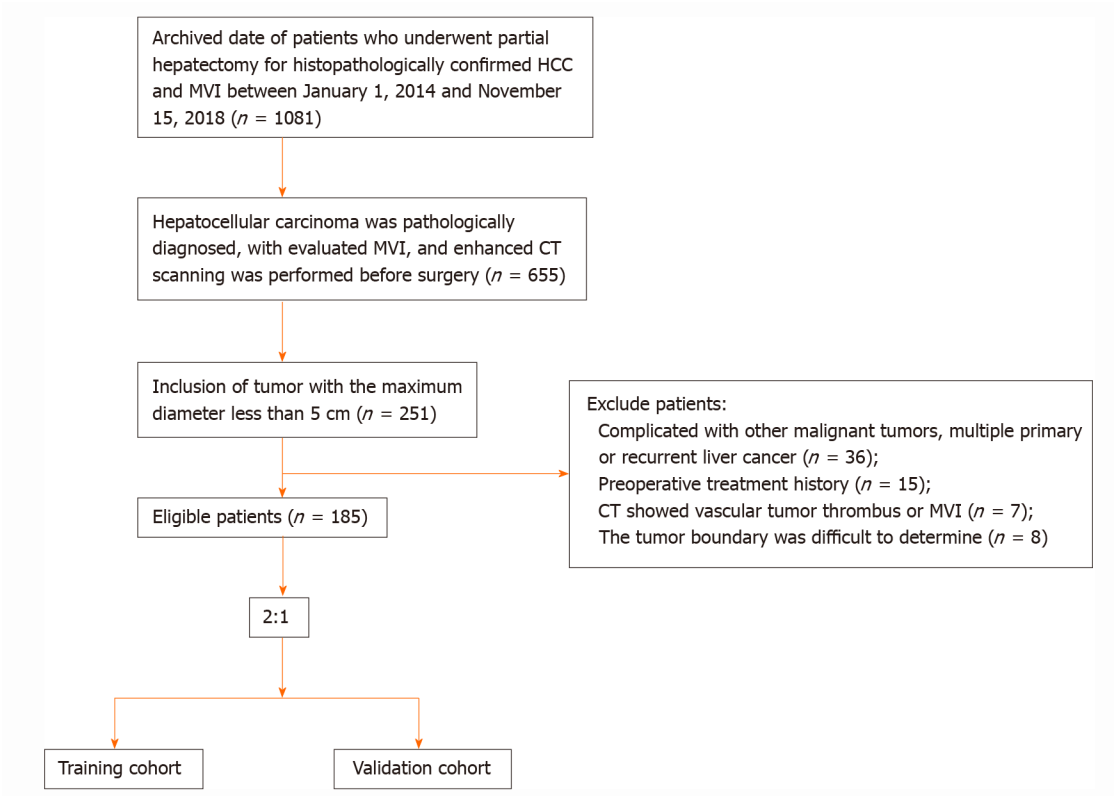


**Figure 1 Screening process for patients with liver cancer.** HCC: Hepatocellular carcinoma; CT: Computed tomography; MVI: Microvascular invasion.

two independent samples was used for categorical variables, and Mann-Whitney *U*-test for two independent samples was used for continuous variables, in order to analyze the difference between the training group and verification group. The receiver operator characteristic curve and the area under the curve (AUC) were used to evaluate the prediction efficiency of the radiomic and image features, and the Delong test was used to determine whether there was a statistical difference between the two methods.

## RESULTS

### *Patient clinicopathologic features*

Finally, the study consisted of 185 patients. Tumor size ranged from 10 mm to 50 mm. The clinicopathological and CT features in the training group and verification group are presented in Table 1. There was no significant difference in scores for MVI, other clinical data, imaging features (TTPVI and RVI), or RS between the two groups ($P > 0.05$), indicating the reasonable arrangement of the training group and verification group.

### *Construction of radiomics tags*

After removal by high correlation, 185 of the 1531 features remained. Then, ten features were selected by dimension reduction using the LASSO algorithm, as shown in Figure 3.

According to the weighting coefficient corresponding to the feature (Figure 3), the radiomics formula was obtained and used to calculate the histological score of each lesion in the training group and verification group. The formula is as follows: Rad-score = -0.66692761 - 0.02491645 × originalshapeFlatness + 0.10564798 × log.sigma.1.0.mm.3DglcmContrast + 0.08789965 × log.sigma.2.0.mm.3DgldmDependenceVariance- 0.06847308 × log.sigma.2.0.mm.3DglszmSizeZoneNonUniformityNormalized - 0.01878760 × log.sigma.2.0.mm.3DglszmSizeZoneNonUniformity + 0.14275995 × log.sigma.3.0.mm.3Dfirstorder90Percentile + 0.04978394 × log.sigma.4.0.mm.3DglszmSmallAreaEmphasis - 0.02095096 × log.sigma.4.0.mm.3DngtdmBusyness + 0.06796763 × wavelet.HLLglcmSumSquares + 0.0767876 × 1wavelet.HLLglszmLargeAreaLow

**Table 1 Clinical and imaging features of patients in the training group and verification group**

| Feature | Training group (*n* = 124) | Verification group (*n* = 61) | *Z* value/$\chi^2$ value | *P* value |
|---|---|---|---|---|
| Age (yr), median (quartile) | 54 (47; 63) | 52 (46; 62) | -0.900 | 0.368 |
| Gender/cases | | | | |
| Male | 102 | 53 | 0.349 | 0.555 |
| Female | 22 | 8 | | |
| Hepatitis B | 104 | 56 | 1.575 | 0.210 |
| Liver cirrhosis | 84 | 43 | 0.044 | 0.833 |
| AFP (ng/mL) | | | | |
| ≤ 20 | 73 | 30 | 1.188 | 0.276 |
| > 20 | 51 | 31 | | |
| MVI | | | | |
| Negative | 82 | 40 | 0.000 | 1.000 |
| Positive | 42 | 21 | | |
| Tumor size (mm), median (quartile) | 36 (28; 44) | 34 (27.5; 41) | -0.746 | 0.456 |
| TTPVI | | | | |
| Negative | 56 | 25 | 0.145 | 0.703 |
| Positive | 68 | 36 | | |
| RVI | | | | |
| Negative | 93 | 40 | 1.362 | 0.243 |
| Positive | 31 | 21 | | |
| Rad-score, median (quartile) | -0.669 (-0.831; -0.546) | -0.640 (-0.780; -0.494) | -0.917 | 0.359 |

AFP: α-fetoprotein; MVI: Microvascular invasion; TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion; Rad-score: Radiomics score.

GrayLevelEmphasis.

### *Predictive performance of radiomics tags*

The median RS [quartile interval] of MVI positive patients [training group: -0.574 (-0.695, -0.412); verification group: -0.495 (-0.644, -0.429)] was significantly higher than that of MVI negative patients [training group: -0.710 (-0.899, -0.610); verification group: -0.709 (-0.805, -0.589)] in both the training and verification groups, and the difference was statistically significant ($P < 0.05$). The radiomics tags exhibited better diagnostic efficacy in both the training and verification groups, as shown in Table 2.

### *Comparison of predictive performance between radiomics tags and MVI-related image features*

As shown in Figure 4 and Table 2, the diagnostic efficacy of the radiomics score was higher than that of the image features in the training group and verification group, and the difference was statistically significant ($P < 0.05$).

## DISCUSSION

The present study revealed that radiomics, as an emerging method at present, exhibited good diagnostic efficiency and better accuracy in predicting MVI, when compared to image features, indicating that radiomics is a more suitable method for predicting MVI in solitary HCC ≤ 5 cm.

A number of studies have shown that tumor size and imaging features can predict MVI, in which TTPVI and RVI are good predictors with an ideal sensitivity and specificity[11]. However, the diagnostic performance of TTPVI and RVI in the present study was significantly lower than that in previous studies, which might be attributed

**Table 2 Comparison of predictive performance between radiomics tags and image features**

| | Training group | | Verification group | |
|---|---|---|---|---|
| | AUC (95%CI) | *P* value | AUC (95%CI) | *P* value |
| Rad-score | 0.724 (0.584-0.863) | | 0.745 (0.655-0.834) | |
| TTPVI | 0.590 (0.500-0.679) | | 0.522 (0.393-0.651) | |
| RVI | 0.545 (0.462-0.628) | | 0.528 (0.401-0.655) | |
| Rad *vs* TTPVI | | 0.018 | | 0.043 |
| Rad *vs* RVI | | 0.002 | | 0.048 |

AUC: Area under the curve; CI: Confidence interval; TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion; Rad-score: Radiomics score.

to the difference in tumor diameter of the study samples. Furthermore, previous studies did not define the tumor size, while the present study merely included patients with a tumor diameter of ≤ 5 cm. In addition, the imaging predictors, such as internal arteries and low-density shadow, were not commonly observed in cases with small tumors, which significantly reduced the positive rate of the MVI-related image features in the present study.

With the recent increase in development of radiomics, numerous studies have indicated that radiomics can reveal the pathological grade, prognosis, MVI, and treatment response of liver cancer. The nomogram of MVI in HCC based on CT radiomics established by Peng *et al*[12] exhibited good decision-making efficiency (AUC = 0.84), which was slightly higher than the results of the present study, but the model did not involve the tumor diameter. In another study[13], the diagnostic efficacy of predicting MVI in a tumor diameter of ≤ 5 cm based on radiomics (the AUC for the verification and verification group was 0.637 and 0.583, respectively) was slightly lower than that of the present study. Compared to that study[13], the present study adopted more stringent inclusion and exclusion criteria. Moreover, the present study only included solitary liver cancer with a diameter of ≤ 5 cm. Partial hepatectomy is the first choice for patients with solitary liver cancer ≤ 5 cm and good liver function[9]. However, if the lesion is not fully resected, the residual MVI near the surgical margin may be an important cause of recurrence in patients with HCC[14], and some studies have demonstrated that extended resection can reduce the early recurrence rate of patients with liver cancer complicated with MVI[15]. Therefore, the present study has certain reference value for surgery choice in patients with liver cancer. In addition, the present study excluded patients with a visible thrombus or the invasion of large blood vessels, rupture and bleeding of liver cancer, and intangible tumor boundary due to other reasons, because the ROI of these patients was difficult to delineate. Hence, measurement errors were hard to avoid. Furthermore, the present study employed the three-dimensional ROI of tumors to the extract radiomics features, which can better reflect the whole outline of the tumor, and allow for the extraction of more tumor information, when compared to two-dimensional ROI. Some studies have revealed that the feature extraction of the maximum cross-sectional area cannot represent the whole tumor[16]. Hence, the present study has obtained more objective prediction results.

The present study had some limitations. First, the present retrospective and single-center study may have selection bias. Second, the present study only used arterial phase images. Multi-phase images may be utilized to obtain more tumor information and improve the diagnostic efficiency. Therefore, multi-center studies with large samples and multi-phase images would become our future research content.

## CONCLUSION

In conclusion, CT radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm. Compared to imaging features, the predictive ability of radiomics tags is significantly higher. The radiomics model of MVI would facilitate clinicians in choosing the appropriate treatment.

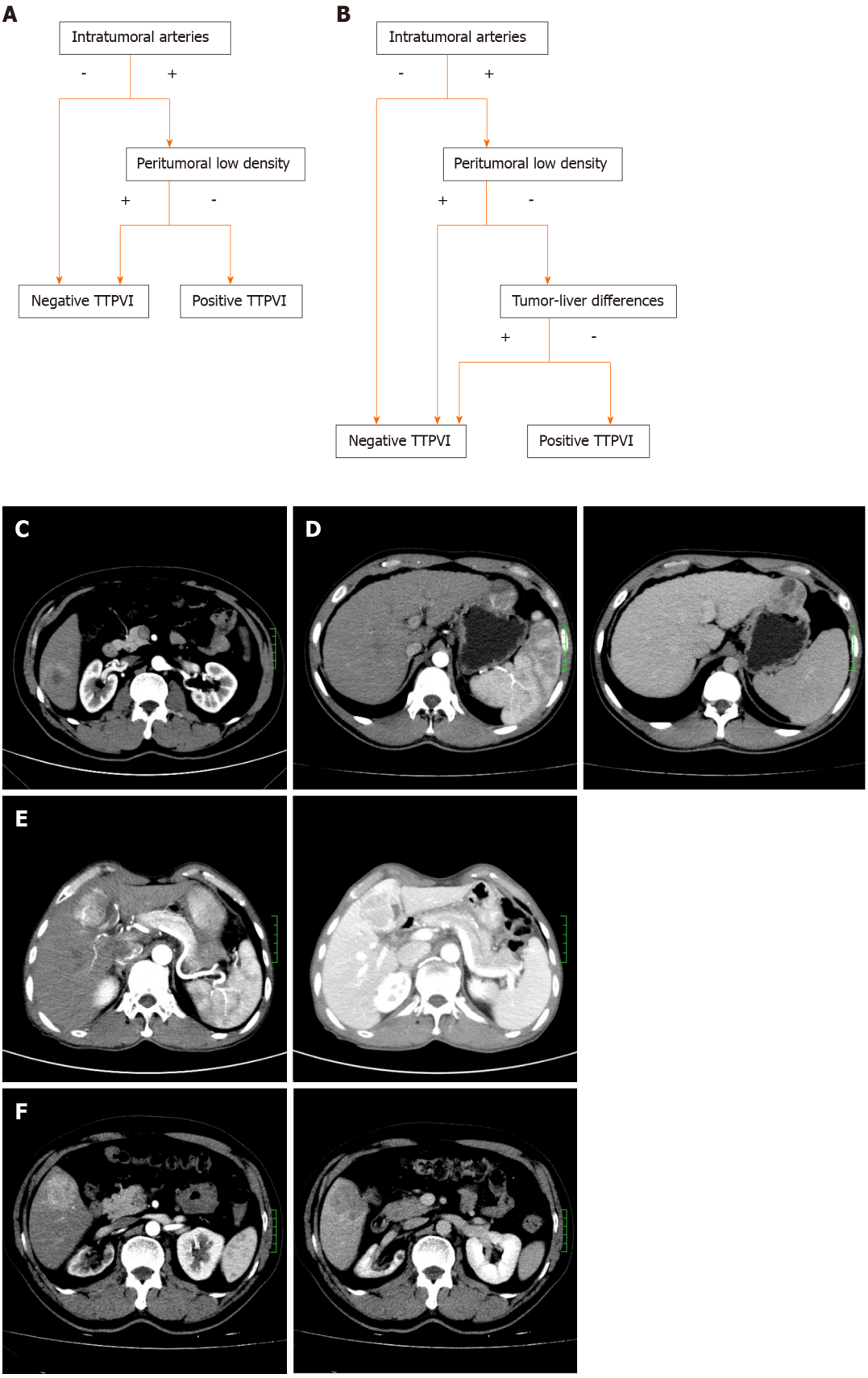


**Figure 2 Specific performance of two-trait predictor of venous invasion and radiogenomic invasion.** A and B: The discriminant process of two-trait predictor of venous invasion (TTPVI) (A) and radiogenomic invasion (RVI) (B); C: Negative intratumoral arteries: Negative TTPVI and RVI; D: Positive intratumoral arteries and peritumoral low density: Negative TTPVI and PVI; E: Positive intratumoral arteries, negative peritumoral low-density shadow, and positive tumor-liver differences: Positive TTPVI and negative RVI; F: Positive intratumoral arteries, negative peritumoral low density, and negative tumor-liver differences: Positive TTPVI and RVI. TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion.

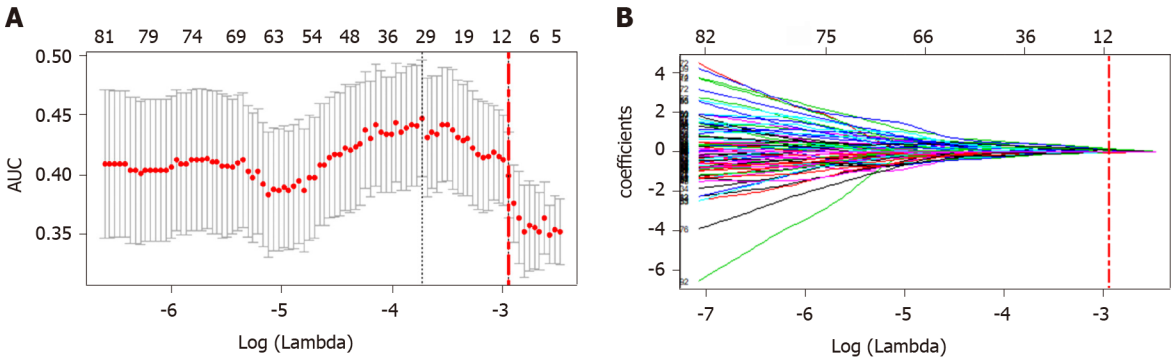


**Figure 3 Selection of radiomic features using the least absolute shrinkage and selection operator-logistic regression model.** A: Coefficient profile of 158 radiomics features against the area under the curve; B: Cross-validation curve. Red dotted vertical lines are drawn at the optimal log (Lambda) by using 10-fold cross-validation and the 1-SE criteria. Ten nonzero coefficients are chosen. AUC: Area under the curve.

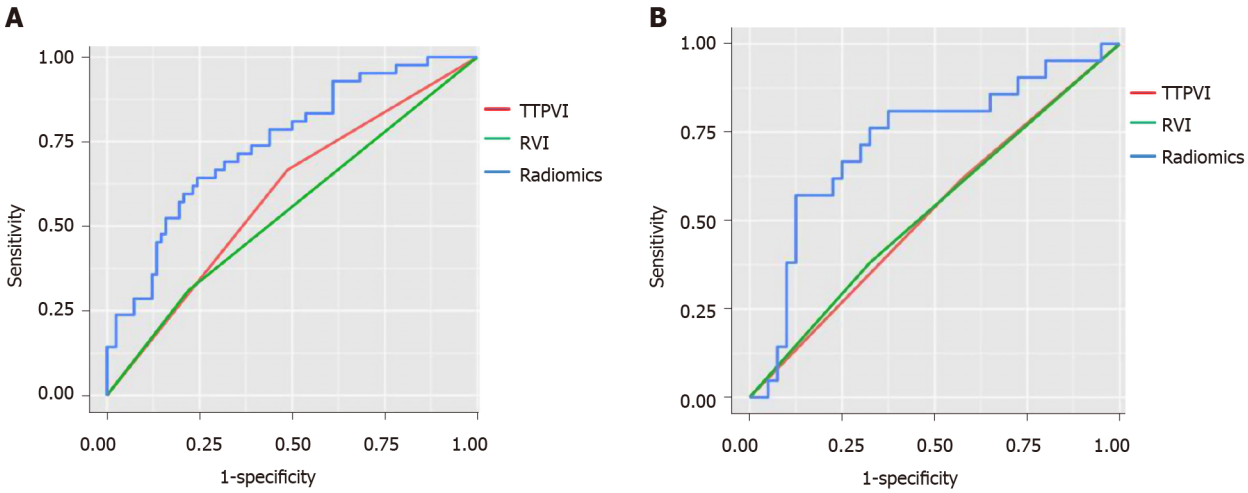


**Figure 4 The two-trait predictor of venous invasion (green curve), radiogenomic invasion (red curve), and receiver operator characteristic of radiomics tag (blue curve) from the two groups.** A: Training group; B: Verification group. TTPVI: Two-trait predictor of venous invasion; RVI: Radiogenomic invasion.

## ARTICLE HIGHLIGHTS

### *Research background*

Liver cancer is one of the most common malignant tumors, and ranks as the fourth leading cause of cancer death worldwide. Microvascular invasion (MVI) is considered one of the most important factors for recurrence and poor prognosis of liver cancer. Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement, and evaluation and better prediction.

### *Research motivation*

At present, few studies have focused on the prediction of MVI in the early stage of hepatocellular carcinoma (HCC) (which refers to solitary tumor with a size of ≤ 5 cm, without MVI). Our study aimed to investigate the predictive value of computed tomography (CT) radiomics for MVI in solitary HCC ≤ 5 cm.

### *Research objectives*

This study aimed to investigate the predictive value of radiomics for MVI in solitary HCC ≤ 5 cm.

### *Research methods*

A total of 185 HCC patients, including 122 MVI negative and 63 MVI positive patients, were retrospectively analyzed. All patients were randomly assigned to the training

group ($n$ = 124) and validation group ($n$ = 61), at a ratio of 2:1. A total of 1351 radiomic features were extracted based on three-dimensional images. In the training group, the least absolute shrinkage and selection operator feature selection algorithm was used to reduce the dimensions, and the most relevant radiomic features of MVI were selected to calculate the image score (Rad-score, RS) of each patient. The diagnostic performance of the radiomics model was verified in the validation group, and the Delong test was applied to compare the radiomics and MVI-related imaging features (two-trait predictor of venous invasion and radiogenomic invasion).

### Research results

A total of ten radiomics features were finally obtained after screening 1531 features. According to the weighting coefficient that corresponded to the features, the RS calculation formula was obtained, and the RS score of each patient was calculated. The radiomics model exhibited a better correction and identification ability in the training and validation groups [area under the curve: 0.72 (95% confidence interval: 0.58-0.86) and 0.74 (95% confidence interval: 0.66-0.83), respectively]. Its prediction performance was significantly higher than that of the image features ($P$ < 0.05).

### Research conclusions

CT radiomics has certain predictive value for MVI in solitary HCC ≤ 5 cm, and the predictive ability is higher than that of image features.

### Research perspectives

The accurate prediction of MVI before surgery is desperately needed. Radiomics as an emerging field, aims to utilize artificial intelligence software to develop methods that may contribute to cancer diagnosis, treatment improvement and evaluation, and better prediction. At present, few studies have focused on the prediction of MVI in the early stage of HCC (which refers to solitary tumor with a size of ≤ 5 cm, without MVI). The present study aimed to investigate the predictive value of CT radiomics for MVI in solitary HCC ≤ 5 cm.

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ORIGINAL ARTICLE

Retrospective Study

# Hepatocellular carcinoma progression in hepatitis B virus-related cirrhosis patients receiving nucleoside (acid) analogs therapy: A retrospective cross-sectional study

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**Author contributions:** Yang DH designed the research study; Yang DH and Wang WP analyzed the data and wrote the manuscript; Zhang Q, Pan HY, Huang YC and Zhang JJ collected the data; All authors have read and approved the final manuscript.

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**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (2020QT155, Hangzhou, Zhejiang Province, China).

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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## Abstract

### *BACKGROUND*

Antiviral therapy cannot completely block the progression of hepatitis B to hepatocellular carcinoma (HCC). Furthermore, there are few predictors of early HCC progression and limited strategies to prevent progression in patients with HBV-related cirrhosis who receive nucleos(t)ide analog (NA) therapy.

### *AIM*

The study aim was to clarify risk factors and the diagnostic value of alpha-fetoprotein (AFP) for HCC progression in NA-treated hepatitis B virus (HBV)-related cirrhosis patients.

### *METHODS*

In this retrospective cross-sectional study, we analyzed the clinical data of 266 patients with HBV-related cirrhosis who received NA treatment between February 2014 and April 2020 at Zhejiang Provincial People's Hospital. The patients were divided into two groups, 145 who did not progress to HCC (No-HCC group), and 121 who progressed to HCC during NA treatment (HCC group). The logistic regression analysis was used to analyze the risk factors of HCC progression. The diagnostic value of AFP for HCC was evaluated by receiver operating characteristic (ROC) curve analysis.

### *RESULTS*

Univariate analysis showed that age ≥ 60 years ($P$ = 0.001), hepatitis B and alcoholic etiology ($P$ = 0.007), smoking history ($P$ < 0.001), family history of HBV-

**Conflict-of-interest statement:** The authors declare that they have no conflicts of interest.

**Data sharing statement:** No additional data are available.



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

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related HCC ($P$ = 0.002), lamivudine resistance ($P$ = 0.011), HBV DNA negative ($P$ = 0.023), aspartate aminotransferase > 80 U/L ($P$ = 0.002), gamma-glutamyl transpeptidase > 120 U/L ($P$ = 0.001), alkaline phosphatase > 250 U/L ($P$ = 0.001), fasting blood glucose (FBG) ≥ 6.16 (mmol/L) ($P$ = 0.001) and Child-Pugh class C ( $P$ = 0.005) were correlated with HCC progression. In multivariate analysis, age ≥ 60 years [hazard ratio (HR) = 3.089, 95% confidence interval (CI): 1.437-6.631, $P$ = 0.004], smoking history (HR = 4.001, 95%CI: 1.836-8.716, $P$ < 0.01), family history of HBV-related HCC (HR = 6.763, 95%CI: 1.253-36.499, $P$ < 0.05), lamivudine resistance (HR = 2.949, 95%CI: 1.207-7.208, $P$ = 0.018), HBV DNA negative (HR = 0.026, 95%CI: 0.007-0.139, $P$ < 0.01), FBG ≥ 6.16 mmol/L (HR = 7.219, 95%CI: 3.716-14.024, $P$ < 0.01) were independent risk factors of HCC progression. ROC of AFP for diagnosis of HCC was 0.746 (95%CI: 0.674-0.818). A cutoff value of AFP of 9.00 ug/L had a sensitivity of 0.609, and specificity of 0.818 for diagnosing HCC.

*CONCLUSION*

Age ≥ 60 years, smoking history, family history of HCC, lamivudine resistance, HBV DNA negative, FBG ≥ 6.16 mmol/L were risk factors of HCC progression. Serum AFP had limited diagnostic value for HCC.

**Key Words:** Hepatitis B virus; Hepatocellular carcinoma; Cirrhosis; Risk factors; Nucleos(t)ide analogs; Progression



**Core Tip:** This retrospective cross-sectional study analyzed risk factors of hepatocellular carcinoma (HCC) progression in hepatitis B virus-related cirrhosis patients receiving nucleoside acid analog therapy for at least 6 mo. We discuss the diagnostic value of serum alpha-fetoprotein level in these patients. The results of the present study increase our understanding of HCC pathogenesis and help to provide HCC prevention and control strategies.

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## INTRODUCTION

Liver cancer has the third-highest cancer mortality rate worldwide[1]. Hepatocellular carcinoma (HCC) is one of the most common subtypes of liver cancer and is the sixth most prevalent cancer type. In most countries, the HCC mortality rates have increased in recent decades[2]. HCC is the fourth most common malignant tumor type in China, accounting for over 55% of the total number of HCC cases[3]. Infection with the hepatitis B virus (HBV) greatly increases the incidence of HCC because HBV causes chronic hepatitis B (CHB), liver cirrhosis, and ultimately HCC[4,5]. The estimated risk of developing HCC was observed to be 25 to 37-fold higher in hepatitis B surface antigen (HBsAg) carriers compared with noninfected patients HBV infection is one of the most important contributors to the pathogenesis of HCC[6-8]. Over the past 30 years, antiviral drugs, especially nucleos(t)ide analogs (NAs), have been widely used in clinical practice and have substantial long-term effects on the inhibition of HBV replication, namely, delaying and reducing the occurrence and development of hepatitis B-related events. Many studies have shown that antiviral therapy can considerably decrease the incidence of HCC, even for patients in whom CHB has progressed to cirrhosis[9,10]. However, antiviral therapy does not completely block the progression of CHB to liver cancer[11,12]. In the current study, we analyzed the risk factors of HCC progression in patients with HBV-related cirrhosis who received

NAs therapy for at least 6 mo. The diagnostic value of the serum alpha-fetoprotein (AFP) level was evaluated in those patients by receiver operating characteristic (ROC) curve analysis. The study results increase our understanding of HCC pathogenesis and help to provide HCC prevention and control strategies.

## MATERIALS AND METHODS

### Patients and design

This cross-sectional study retrospectively enrolled 266 patients with HBV-related cirrhosis who were treated with NA antiviral therapy at Zhejiang Provincial People's Hospital between February 2014 and April 2020. The 266 patients were divided into two groups, 145with cirrhosis who did not progress to HCC during the observation period (No-HCC group), and 121 with cirrhosis who progressed to HCC (HCC group). The inclusion criteria were: (1) Age ≥ 18 years; (2) Treatment with lamivudine (LAM), adefovir (ADV), telbivudine, entecavir (ENT), or tenofovir (TDF) nucleoside or NAs for at least 6 mo; (3) Diagnosis of cirrhosis established by either histology (progressive fibrosis, nodule formation, and loss of hepatic architecture) or clinical data (symptoms and signs of cirrhosis, abnormal liver function, and ultrasonic identification). Demographic, clinical, laboratory, imaging, and pathology data were collected during the patient's hospital stay. The severity of cirrhosis was classified by the Child-Pugh criteria. Patients with HBV-related cirrhosis were diagnosed in accord with the guidelines for the prevention and treatment of chronic hepatitis B formulated by the Hepatology Branch and the Infectious Diseases Branch of the Chinese Medical Association[13,14]; and (4) HCC and hepatitis diagnoses confirmed by clinical and serological characteristics, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), hepatic arteriography with digital subtraction angiography (DSA) and pathological examination consistent with the hepatitis and primary liver cancer clinical diagnosis criteria[15,16]. The exclusion criteria were: (1) Hepatitis C virus, hepatitis D virus, or human immunodeficiency virus coinfection; (2) Autoimmune hepatitis and drug hepatitis; and (3) Hepatocarcinoma prior to antiviral treatment or within 6 mo after antiviral treatment. The study received no support from any pharmaceutical company and was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (2020QT155), Hangzhou, Zhejiang Province, China.

### Data collection and study design

The data collected from the electronic medical record system were age, sex, history of drinking and smoking, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), total bilirubin, fasting blood glucose (FBG), AFP, prothrombin time (PT), presence of ascites, hepatic encephalopathy, Child-Pugh score and classification, and family history of HBV, hepatitis B, and HCC. Serological markers of hepatitis B, serum content of HBV DNA, and history of NA treatment were reported by the patients or their families. All patients in the cohort were followed-up for 3 years or until death.

Serum liver function was tested by routine automated techniques using an Olympus AU5400 automated analyzer (Olympus, Tokyo, Japan). HBsAg, hepatitis B e-antigen (HBeAg), and hepatitis B e-antibody were assessed at baseline by chemiluminescence immunoassay (Abbott ARCHITECT i2000 SR analyzer; Abbott Diagnostics, Chicago, IL, United States). The serum HBV DNA load was assessed by RT-PCR using a LightCycler PCR system (Roche LightCycler480 II fluorescence quantitative PCR) in strict accordance with the instructions provided with the reagent kit (Shenzheng PG Biotech Co. Ltd, China). The detection limit was approximately 100 viral genome IU/mL. Genotypic resistance to LAM and ADV were determined at baseline by direct sequencing of the PCR amplification products. The serum AFP tumor marker was assayed by electrochemiluminescence. The study protocol conformed to the 1975 Declaration of Helsinki ethical guidelines for clinical studies.

### Statistical analysis

Descriptive data were expressed as means ± SD or $n$ (%). Continuous variables were compared using Student's $t$-test. Skewness distribution data were reported as the median with the range and were analyzed using the Mann–Whitney $U$ test. Categorical variables were analyzed using Fisher's exact test or Pearson's $\chi^2$ test. A logistic regression model was used to analyze single factors, and multivariate analysis with stepwise regression was used to identify statistically significant variables in the

single-factor analysis. The diagnostic performance of the serum AFP level was evaluated using ROC curves. The cutoff value, which was the maximum area under the ROC curve (AUROC), and accuracy were calculated with 95% confidence intervals (CIs). A *P* value of less than 0.05 was determined to indicate statistical significance. The SPSS statistical package (version 22.0; SPSS, Chicago, IL, United States) was used for the statistical analysis.

## RESULTS

### *Clinical characteristics of HBV-related cirrhosis patients receiving NA therapy*

The clinical features of the No-HCC and the HCC group were compared. There were no significant differences between the two groups in the sex ratio, duration of NA therapy, HBsAg level, HBeAg positivity, jaundice index, Child-Pugh class B ratio, and PT level ($P > 0.05$). The HCC group included significantly more patients older than 60 years of age and patients with increased levels of ALT, AST, GGT, ALP, and FBG, and decreased levels of ALB than the No-HCC group. The HCC group also contained more patients with mixed etiology (alcohol + HBV), history of smoking, family history of HBV-related HCC, LAM resistance, Child-Pugh class C status, and an AFP > 20 μg/L and fewer HBV DNA-negative and Child-Pugh class A patients than the No-HCC group (Table 1).

### *Risk factors for HCC progression in HBV-related cirrhosis patients receiving NA therapy*

We analyzed factors associated with HCC progression in HBV-related cirrhosis patients who received NA therapy. Univariate analysis found age ≥ 60 years ($P = 0.002$), HBV + alcohol mixed etiology ($P = 0.007$), smoking history ($P < 0.001$), family history of HBV-related HCC ($P = 0.002$), LAM resistance ($P = 0.046$), HBV DNA negativity ($P = 0.023$), AST > 80 U/L ($P = 0.002$), GGT > 120 U/L ($P = 0.001$), ALP > 250 U/L ($P = 0.001$), FBG ≥ 6.16 mmol/L ($P = 0.001$), and Child-Pugh class C ($P = 0.005$) to be significantly related to HCC (Table 2). Multivariate analysis showed that age ≥ 60 years [hazard ratio (HR) = 3.089, 95%CI: 1.437-6.631, $P = 0.004$], smoking history (HR = 4.001 95%CI: 1.836-8.716, $P < 0.01$), family history of HBV-related HCC (HR = 6.763, 95%CI: 1.253-36.499, $P < 0.05$), LAM resistance (HR = 2.949, 95%CI: 1.207-7.208, $P = 0.018$), HBV DNA negative (HR = 0.026, 95%CI: 0.007-0.139, $P < 0.01$), and FBG ≥ 6.16 mmol/L (HR = 7.219, 95%CI: 3.716-14.024, $P < 0.01$) independently predicted HCC progression in patients with HBV-related cirrhosis who received NAs therapy (Table 3).

### *Serum AFP levels in the No-HCC and HCC groups*

In the HCC group, there were 56 patients with serum AFP levels > 20 μg/L, but that was seen in only 17 patients in the No-HCC group ($P < 0.001$). In the HCC group, 65 patients had AFP levels < 20 μg/L. The AFP levels of 37 patients in the HCC group were > 400 μg/L, but only two patients in the No-HCC group levels > 400 μg/L ($P < 0.001$, Table 4). The 17 patients in the No-HCC group with AFP levels > 20 μg/L were followed for 1 year. Their AFP level was determined every month and showed a dynamic decline, returning to normal within 1 year. All patients underwent enhanced MRI of imaging of the liver. We believe that hepatitis B activity, rather than HCC, led to the AFP abnormality. The AFP level distributions in the two groups are shown in Figures 1 and 2.

### *Serum AFP has limited ability to diagnose HBV-related HCC*

We investigated the value of using serum AFP to diagnose HCC in patients who had HBV-related cirrhosis and were receiving NAs therapy. The AUROC of serum AFP for the diagnosis of HCC was 0.746 (95%CI: 0.674-0.818). The sensitivity of serum AFP in diagnosing HCC in those patients was 0.609, and the specificity was 0.818. The positive predictive value of HCC was 22.51%, the negative predictive value of HCC was 46.07%, the cutoff was 9.00, and the Youden index was 0.427 (Figure 3).

## DISCUSSION

HBV infection remains a major risk factor for the development of cirrhosis and HCC[17]. Patients with chronic HBV are at risk of developing liver-related complica-

**Table 1 Comparison of the clinical characteristics of patients with and without hepatocellular carcinoma**

| Characteristic | no-HCC group (*n* = 145) | HCC group (*n* = 121) | *P* value |
|---|---|---|---|
| Age ≥ 60 yr, *n* (%) | 41 (28.3) | 64 (52.9) | < 0.001 |
| Male, *n* (%) | 99 (68.3) | 91 (75.2) | 0.133 |
| Etiology of liver cirrhosis, *n* (%) | | | |
| HBV | 101 (60.7) | 76 (62.8) | 0.363 |
| HBV + alcohol | 31 (21.4) | 45 (37.2) | 0.006 |
| HBV + HEV | 1 (0.6) | 0 (0.0) | - |
| HBV + schistosome | 1 (0.6) | 0 (0.0) | - |
| Smoking history, *n* (%) | 21 (14.5) | 51 (42.1) | < 0.001 |
| Family history of HBV-related HCC, *n* (%) | 10 (6.9) | 23 (19.0) | 0.004 |
| Medication history | | | |
| Duration of NA treatment, yr, median ($P_{25}$, $P_{75}$) | 3.9 (2.1, 5.8) | 5.4 (2.3, 6.9) | 0.067 |
| LAM resistance, *n* (%) | 18 (12.4) | 27 (22.3) | 0.021 |
| HBsAg level, IU/L, median ($P_{25}$, $P_{75}$) | 255.0 (56.0, 678.0) | 269.0 (67.0, 656.0) | 0.456 |
| HBeAg positive, *n* (%) | 37 (25.5) | 21 (17.4) | 0.136 |
| HBV DNA negative, *n* (%) | 67 (46.2) | 39 (32.2) | 0.033 |
| ALB, U/L | 36.29 ± 7.98 | 33.34 ± 6.62 | 0.002 |
| ALT, U/L, median ($P_{25}$, $P_{75}$) | 27.00 (18.00, 37.00) | 32.00 (21.27, 62.00) | 0.006 |
| AST, U/L, median ($P_{25}$, $P_{75}$) | 33.00 (23.00, 47.00) | 44.50 (31.65, 96.85) | < 0.001 |
| GGT, U/L, median ($P_{25}$, $P_{75}$) | 33.00 (20.00, 46.00) | 61.50 (32.75, 160.75) | < 0.001 |
| ALP, U/L, median ($P_{25}$, $P_{75}$) | 99.00 (73.00, 126.00) | 134.50 (92.00, 198.85) | < 0.001 |
| TB, μmol/L, median ($P_{25}$, $P_{75}$) | 20.41 (13.81, 44.60) | 24.46 (16.60, 42.80) | 0.192 |
| FBS, mmol/L, median ($P_{25}$, $P_{75}$) | 5.17 ± 0.68 | 6.99 ± 1.31 | 0.025 |
| Ascites, *n* (%) | 33 (20.0) | 43 (34.7) | 0.022 |
| Child-Pugh class, *n* (%) | | | |
| A | 81 (55.9) | 55 (45.5) | 0.001 |
| B | 38 (26.2) | 26 (21.5) | 0.561 |
| C | 26 (17.9) | 40 (33.1) | 0.002 |
| AFP ≥ 20 μg/L, *n* (%) | 14 (9.7) | 56 (46.3) | < 0.001 |
| PT s, median ($P_{25}$, $P_{75}$) | 13.25 (11.80, 14.20) | 13.51 (12.41, 15.02) | 0.475 |

AFP: Alpha-fetoprotein; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FBS: Fasting blood sugar; GGT: Glutamyl transpeptidase; HBeAb: Hepatitis B e-antibody; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: Lamivudine; PT: Prothrombin time; SD: Standard deviation; TB: Total bilirubin.

tions, namely, cirrhosis and HCC. In China, 77% of cirrhosis cases and 84% of HCC cases are caused by HBV infection[18]. Antiviral therapy can reduce but not eliminate the risk of developing HCC[19-21]. The annual incidence of HCC ranges from 0.01% to 5.4% in patients with CHB who are treated with ENT or TDF[19,22]. NAs antiviral treatments can markedly inhibit viral replication and improve liver necrosis, inflammation, and fibrosis. However, NAs cannot eliminate covalently closed template DNA (cccDNA) produced during hepatitis B viral replication or clear the integrated HBV genome; therefore, NAs cannot completely block hepatitis B cirrhosis from developing into HCC[12,21]. The persistence of cccDNA and an integrated HBV genome is the basis for hepatitis B cirrhosis progressing to primary liver cancer[23,24]. In this study, we investigated the clinical characteristics of HCC progression in patients with HBV-related cirrhosis who received antiviral therapy with NAs.

**Table 2 Univariate logistic regression analysis of hepatitis B-related cirrhosis progressing to hepatocellular carcinoma in patients treated with nucleos(t)ide analogs**

| Characteristic | No-HCC group (*n* = 145) | HCC group (*n* = 121) | Univariate adjusted HR (95%CI) | *P* value |
|---|---|---|---|---|
| Age, yr | | | | |
| ≥ 60 | 40 (27.6) | 65 (52.9) | 2.664 (1.606-4.418) | 0.001 |
| < 60 | 105 (72.4) | 56 (46.3) | | |
| HBV + alcohol | | | | |
| Yes | 31 (21.4) | 45 (37.2) | 2.384 (1.271-4.473) | 0.007 |
| No | | | | |
| Smoking history | | | | |
| Yes | 21 (14.5) | 51 (42.1) | 4.073 (2.281-7.273) | < 0.001 |
| No | | | | |
| Family history of HBV-related HCC | | | | |
| Yes | 10 (6.9) | 23 (19.0) | 3.546 (1.573-7.998) | 0.002 |
| No | | | | |
| LAM resistance | | | | |
| Yes | 18 (12.4) | 27 (22.3) | 2.284 (1.214-4.297) | 0.011 |
| No | | | | |
| HBV DNA negative | | | | |
| Yes | 67 (46.2) | 39 (32.2) | 0.559 (0.339-0.922) | 0.023 |
| No | | | | |
| ALB (g/L) | | | | |
| < 35 | 67(46.2) | 62(51.2) | 1.223 (0.754-1.984) | 0.414 |
| ≥ 35 | 78 (53.8) | 59 (48.8) | | |
| ALT (U/L) | | | | |
| 50-100 | 13 (9.0) | 15 (12.4) | 1.324 (0.612-2.866) | 0.476 |
| > 100 | 10 (7.0) | 14 (11.6) | 1.138(0.482-2.688) | 0.768 |
| AST (U/L) | | | | |
| 40-80 | 32 (22.1) | 24 (19.8) | 0.919 (−0.713-0.514) | 0.783 |
| > 80 | 16 (10.3) | 31 (29.8) | 2.899 (0.436-1.767) | 0.002 |
| GGT (U/L) | | | | |
| 60-120 | 15 (10.3) | 20 (16.5) | 1.853 (0.892-3.847) | 0.098 |
| > 120 | 8 (5.1) | 28 (23.1) | 5.663 (1.075-2.573) | 0.001 |
| ALP (U/L) | | | | |
| 125-250 | 36(24.8) | 45 (38.8) | 1.609 (−0.062-1.028) | 0.073 |
| > 250 | 6 (1.4) | 21 (17.4) | 4.865 (0.667-2.993) | 0.001 |
| FBG (mmol/L) | | | | |
| ≥ 6.16 | 19 (13.1) | 37 (30.6) | 3.3179 (0.587-1.902) | 0.001 |
| < 6.16 | | | | |
| Ascites class | | | | |
| Yes | 33 (22.8) | 43 (35.5) | 0.834 (−0.412-0.060) | 0.142 |
| No | | | | |

| | | | | |
|---|---|---|---|---|
| Child-Pugh class | | | | |
| A | 81 (58.6) | 55 (37.9) | 0.658 (−0.938-0.064) | 0.091 |
| B | 38 (26.2) | 26 (21.5) | 0.671 (−0.981-0.112) | 0.165 |
| C | 26 (17.9) | 40 (33.1) | 2.260 (0.247-1.427) | 0.005 |

Data are *n* (%) or mean ± SD as shown. AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; FBG: Fasting blood glucose; GGT: Glutamyl transpeptidase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HR: Hazard ratio; LAM: Lamivudine. .

**Table 3 Multivariate analysis of factors associated with hepatitis B-related cirrhosis progression to hepatocellular carcinoma in nucleos(t)ide analog-treated patients**

| Risk factor | β | SE | Wald | *P* value | OR (95%CI) |
|---|---|---|---|---|---|
| Age ≥ 60 yr | 1.127 | 0.390 | 8.347 | 0.004 | 3.089 (1.437-6.631) |
| Smoking history | 1.387 | 0.397 | 12.180 | < 0.01 | 4.001 (1.836-8.716) |
| Family history of HBV-related HCC | 1.911 | 0.860 | 4.938 | < 0.05 | 6.763 (1.253-36.499) |
| LAM resistance | 1.082 | 0.456 | 5.638 | 0.018 | 2.949 (1.207-7.208) |
| HBV DNA negative | -3.479 | 0.816 | 19.427 | < 0.01 | 0.026 (0.007-0.139) |
| FBG ≥ 6.16 mmol/L | 1.977 | 0.339 | 34.030 | < 0.01 | 7.219 (3.716-14.024) |

CI: Confidence interval; FBG: Fasting blood glucose; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: Lamivudine OR: Odds ratio;; SE: Standard error.

**Table 4 Comparison of the alpha-fetoprotein level distributions in patients with and without hepatocellular carcinoma, *n* (%)**

| AFP (μg/L) | No-HCC group (*n* = 145) | HCC group (*n* = 121) | *P* value |
|---|---|---|---|
| < 20 | 128 (88.27) | 65 (53.72) | < 0.001 |
| 20-100 | 12 (8.27) | 14 (11.57) | 0.51 |
| 100-200 | 1 (0.69) | 2 (2.17) | 0.231 |
| 200-400 | 2 (1.38) | 3 (2.48) | 1 |
| ≥ 400 | 2 (1.38) | 37 (30.58) | < 0.001 |

AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

Medical and healthcare progress, improved living standards, and decreased population fertility have led to population aging in China. Data from 22 tumor registration centers in China have shown that the average age of liver cancer onset increased from 58.80 to 62.35 years for men and from 64.02 to 68.99 years for women between 2000 and 2014[25]. Our study found that age ≥ 60 years was an independent risk factor for the progression of hepatitis B-related cirrhosis to HCC while receiving NAs antiviral therapy. An aging population and the burden caused by HCC mortality could be a future challenge for China. Previous studies have confirmed that poor lifestyle habits are related to a high incidence of liver cancer; in particular, a history of smoking and drinking increases the risk of HCC[26,27]. Tobacco smoke contains various carcinogens, 11 of which are classified as International Agency for Research on Cancer Group 1 human carcinogens. Epidemiologic evidence from a recent meta-analysis showed a positive association between current tobacco smoking and liver cancer risk (risk ratio: 1.55, 95%CI: 1.46-1.65), suggesting a causal role of smoking in liver cancer development[28]. Liu *et al*[29] found that tobacco smoking and HBV infection positively interact in the development of liver cancer. Our results revealed smoking to be an independent risk factor of HCC progression (95%CI: 1.836-8.716, $P < 0.01$) even if the patients were receiving antiviral treatment. Studies have frequently reported that a family history of liver cancer increases HCC risk independent of

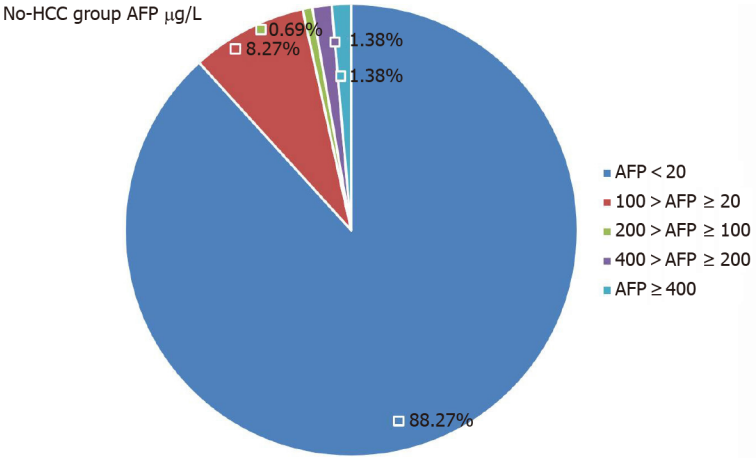


**Figure 1 Distribution of alpha-fetoprotein levels in patients without hepatocellular carcinoma.** AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

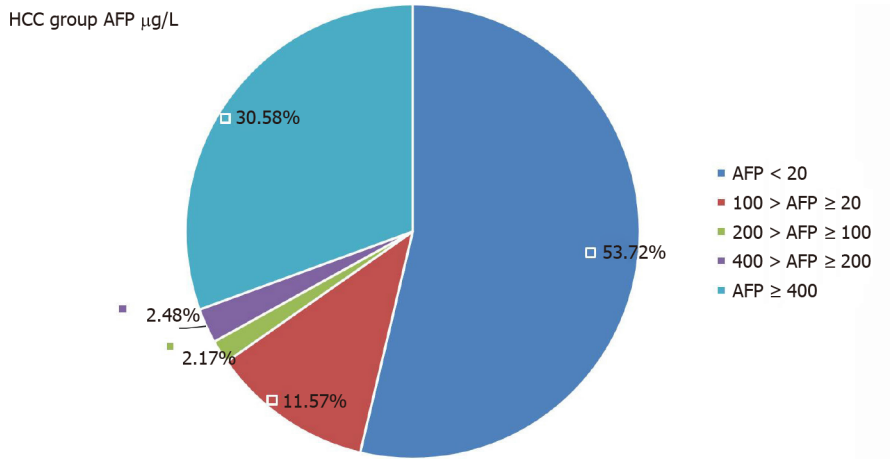


**Figure 2 Distribution of alpha-fetoprotein level in patients with hepatocellular carcinoma.** AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

hepatitis. The combination of a family history of liver cancer and hepatitis B serum markers is associated with a greater than 70-fold increase in HCC risk[30]. Super-additive and super-multiplicative interactions may exist between a family history of liver cancer and HBV infection that increase the risk of the development of liver cancer[31]. Our study found that a family history of HBV-related HCC is a risk factor of progression in cirrhotic patients receiving antiviral therapy (95%CI: 1.253-36.499, $P < 0.05$). Our results suggest that patients with HBV-related cirrhosis who smoke should quit smoking and cultivate a healthy lifestyle to reduce the risk of developing HCC[32,33]. Furthermore, close monitoring should be carried out if a patient's first-degree relative develops HBV-related HCC during antiviral treatment.

LAM has been used as an antiviral treatment for hepatitis B for the past 20 years in China. Some Chinese patients with hepatitis B-related cirrhosis have previously undergone primary LAM monotherapy. Studies have shown that long-term LAM antiviral therapy can delay disease progression, reduce liver function decompensation, and reduce the incidence of HCC[34,35]. The inflammation seen in liver histology can improve in patients with hepatitis B-related cirrhosis when they are treated with LAM, but with a prolonged treatment time, the incidence of drug-resistant viral mutation increases[34,36]. The clinical benefit of LAM is limited by the emergence of resistant mutant strains and viral breakthrough. Although some LAM-resistant CHB patients have received ADV combination therapy or sequential ENT or TDF monotherapy, some of these patients did not experience a beneficial treatment effect and still had continuous replication of HBV in the liver[37,38]. In patients with LAM resistance, those with cirrhosis had a higher HCC risk than non-cirrhotic patients. Rescue treatment with ADV in patients who developed viral breakthrough did not appear to reduce the risk of HCC compared with untreated patients without remission[11]. In

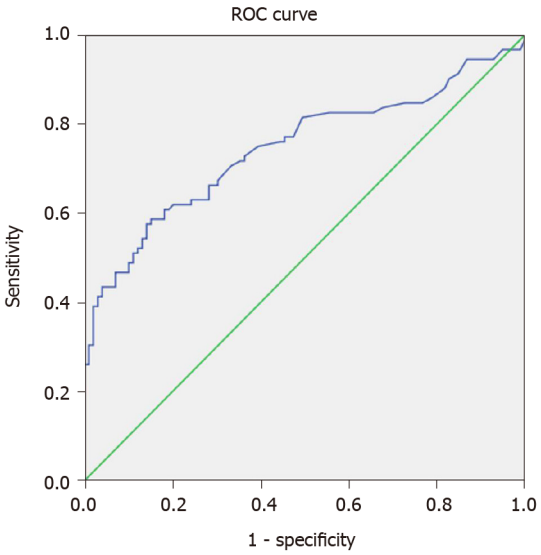


**Figure 3 Receiver operating characteristic curve analysis of alpha-fetoprotein to diagnose hepatocellular carcinoma in patients with hepatitis B virus-related cirrhosis receiving nucleos(t)ide analog therapy.** ROC: Receiver operating characteristic.

our study of patients treated with antiviral therapy for more than 6 mo, the number of HBV DNA-negative patients in the HCC group was lower than that in the No-HCC group. Many studies have found a correlation between serum HBV DNA levels and the occurrence of HCC in patients with hepatitis B. Kaneko *et al*[39] reported that detectable HBV DNA was significantly associated with a higher risk of HCC development compared with continuously undetectable HBV DNA. Chen *et al*[40] found that the incidence of HCC increased with serum HBV DNA level at study entry in a dose-response relationship ranging from 108/100,000 person-years for an HBV DNA level of < 300 copies/mL to 1152/100,000 person-years for an HBV DNA level of 1 million copies/mL or more. The corresponding cumulative incidence rates of HCC were 1.3% and 14.9%.

Even if the serum HBV DNA level in patients with hepatitis B-related cirrhosis is kept at a low level (< 2000 IU/mL), the risk of HCC is still high[41]. As shown in our study, HBV DNA negativity (HR = 0.026, 95%CI: 0.007-0.139, $P < 0.01$) independently predicted HCC progression in patients with HBV-related cirrhosis who received NAs therapy. Thus, to help avoid HCC progression, hepatitis B patients should continue to maintain an HBV DNA-negative status. Therefore, drugs with a high genetic barrier to resistance are suggested as first-line antiviral drugs for HBV therapy, and are recommended by current guidelines. Drugs such as ETV, TDF, and TDF alafenamide fumarate should be selected to generate a sustained antiviral treatment response and to reduce the occurrence of HBV resistance and the incidence of HCC[42,43].

As an important metabolic organ, the liver plays a key role in maintaining glucose homeostasis. Studies have found a positive relationship between liver cancer and diabetes mellitus[44,45]. Cell and animal experiments have shown that type 2 diabetes and male sex are associated with HCC development. Gao *et al*[46] demonstrated that heterozygous deletion of the *Ncoa5* gene caused spontaneous development of HCC exclusively in male mice, and NCOA5 deficiency increased susceptibility to both glucose intolerance and HCC. In a prospective cohort study, adjusted multivariable analysis showed that participants with 4.82 mmol/L ≤ FBG ≤ 5.49 mmol/L had a 47% increased risk of HCC, and those with an FBG > 5.49 mmol/L had a 69% increased risk[47]. In our study, the FBG level in the HCC group was higher than that in the No-HCC group (Table 1), and FBG ≥ 6.16 mmol/L was an independent risk factor for the HCC progression in patients with hepatitis B-related cirrhosis receiving NA antiviral treatment. Controlling blood sugar concentrations might be a way to decrease the risk of HCC in the Chinese population.

AFP is a glycoprotein that exists in a variety of different glycotypes. AFP has been used in the screening, diagnosis, efficacy evaluation, and prognosis evaluation of HCC. AFP elevation is commonly seen in active hepatitis, pregnancy, liver cancer, and embryonic tumors[48,49]. Previous studies reported that there was a significant correlation between serum AFP levels and the tumor size in liver cancer, and that the sensitivity and specificity of AFP depended on the selected serum level threshold[20,50]. Liu *et al*[51] found that approximately one-third of patients with

HCC had normal serum AFP levels and that the level was related to the volume of liver cancer lesions, vascular invasion, and differentiation. In our study, we found 56 patients with a serum AFP > 20 μg/L, and 19 with serum AFP levels between 20 and 400 μg/L and with HCC confirmed by imaging and histopathological examination of liver masses. However, an AFP level of < 20 μg/L did not exclude the possibility of HCC. This study showed that 65 patients with a serum AFP of < 20 μg/L had space-occupying lesions that were confirmed as HCC by MRI enhancement, histopathology, and liver DSA examination. That indicates that more sensitive diagnostic markers of HCC must be developed. In addition, more sensitive serum tumor markers such as milk fat globule-EGF factor 8, osteopontin, miRNA classifier, glypican-3[52-54], and others should be actively investigated or combined to identify HCC at an early stage[55]. However, their clinical sensitivity and specificity must first be confirmed.

This study had some limitations. First, it was not prospective. The impact of antiviral treatment time and the amount of smoking and drinking on the development of HCC require confirmation in prospective studies with larger sample sizes. Second, longer follow-up and surveillance of HBV-related cirrhosis patients receiving NA therapy is necessary to observe whether they progress to HCC in their lifetime, even though the 145 cirrhotic patients with NA therapy did not progress to HCC during the observation period. Third, additional molecular markers should be assessed for their ability to provide an early diagnosis of HCC in patients with HBV-related cirrhosis. Some data indicate that the currently used potent NAs can reduce but not eliminate the risk of HCC. The inability to eliminate HCC risk might persist because of risk factors that are not amenable to change by antiviral therapy or because of events that may have taken place before treatment initiation.

## CONCLUSION

In conclusion, age ≥ 60 years, a history of smoking, family history of HBV-related HCC, LAM resistance, HBV DNA negativity, and FBG ≥ 6.16 mmol/L were risk factors for HCC progression in patients with HBV-related cirrhosis who received NAs therapy. Patients with HBV-related cirrhosis should be treated with NA antiviral therapy that has a high genetic barrier to resistance[7] in order to improve the long-term response to antiviral therapy, to maintain an HBV DNA-negative status, and to prevent subsequent hepatitis activity. The early identification of HCC in patients with HBV-related cirrhosis remains difficult. Patients who receive antiviral therapy with NAs, especially those older than 60 years of age, should avoid smoking, control their blood sugar at a reasonable level, and undergo routine imaging examination of liver biochemistry and serum AFP evaluation. If space-occupying lesions are identified, the patient should undergo liver CT or MRI enhancement, or even liver DSA examination to identify HCC as promptly as possible, even if the AFP level is within the normal range.

## ARTICLE HIGHLIGHTS

### *Research background*

Antiviral therapy cannot completely block the progression of hepatitis B to hepatocellular carcinoma (HCC). Furthermore, there are few early predictors of HCC progression and early identification is difficult in patients with HBV-related cirrhosis who receive nucleos(t)ide analog (NA) therapy. The study is helpful to provide HCC prevention and control strategies by analyzing the risk factors of HCC progression and the diagnostic value of AFP for HCC in those people.

### *Research motivation*

The study objective was to identify factors that affect the occurrence of HCC and how to identify early HCC in patients with hepatitis B virus (HBV)-related cirrhosis who receive NA therapy. The results can improve the understanding of the development of HCC in those patients so as to improve the early detection and prevention of HCC.

### *Research objectives*

The study objectives were to clarify risk factors and the diagnostic value of alpha-fetoprotein (AFP) for HCC progression in patients with HBV-related cirrhosis treated with NAs and to provide new strategies for prevention and control of HCC in those

patients.

### Research methods

Logistic regression analysis was used to analyze the risk factors of HCC progression. The diagnostic value of AFP for HCC was evaluated by receiver operating characteristic curve analysis.

### Research results

The study showed that age ≥ 60 years, smoking history, family history of HCC, lamivudine resistance, HBV DNA negativity, fasting blood sugar ≥ 6.16 mmol/L were independent risk factors of HCC progression. Serum AFP had limited diagnostic value for HCC. The results provide a meaningful strategy for early prediction and identification for HCC in those patients.

### Research conclusions

A retrospective cross-sectional study was conducted to analyze risk factors of HCC progression in HBV-related cirrhosis patients receiving NA therapy. Metabolic effects of fasting blood sugar levels on the progress of HCC were seen during the receipt of NA therapy. The diagnostic value of the serum AFP level was evaluated in those patients.

### Research perspectives

The study results will change the strategies used to prevent HCC in patients with HBV-related cirrhosis an receive NA therapy.

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ORIGINAL ARTICLE

**Observational Study**

# Hypophosphatemia after high-dose intravenous iron treatment in patients with inflammatory bowel disease: Mechanisms and possible clinical impact

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## Abstract

### *BACKGROUND*

High-dose intravenous iron is an effective treatment option for iron deficiency (ID) or ID anaemia (IDA) in inflammatory bowel disease (IBD). However, treatment with ferric carboxymaltose (FCM) has been associated with the development of hypophosphatemia.

### *AIM*

To investigate mechanisms behind the development of hypophosphatemia after intravenous iron treatment, and disclose symptoms and clinical manifestations related to hypophosphatemia short-term.

### *METHODS*

A prospective observational study of adult IBD patients with ID or IDA was conducted between February 1, 2017 and July 1, 2018 at two separate university hospitals in the southeast region of Norway. Patients received one dose of 1000

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mg of either FCM or ferric derisomaltose (FDI) and were followed for an observation period of at least 7 wk. Blood and urine samples were collected for relevant analyses at baseline, week 2 and at week 6. Clinical symptoms were assessed at the same timepoints using a respiratory function test, a visual analogue scale, and a health-related quality of life questionnaire.

*RESULTS*

A total of 106 patients was available for analysis in this study. The FCM treatment group consisted of 52 patients and hypophosphatemia was present in 72.5% of the patients at week 2, and in 21.6% at week 6. In comparison, the FDI treatment group consisted of 54 patients and 11.3% of the patients had hypophosphatemia at week 2, and 3.7% at week 6. The difference in incidence was highly significant at both week 2 and 6 ($P < 0.001$ and $P < 0.013$, respectively). We observed a significantly higher mean concentration of intact fibroblast growth factor 23 ($P < 0.001$), a significant rise in mean urine fractional excretion of phosphate ($P = 0.004$), a significant decrease of 1,25-dihydroxyvitamin D ($P < 0.001$) and of ionised calcium levels ($P < 0.012$) in the FCM-treated patients compared with patients who received FDI. No clinical symptoms could with certainty be related to hypophosphatemia, since neither the respiratory function test, SF-36 (36-item short form health survey) or the visual analogue scale scores resulted in significant differences between patients who developed hypophosphatemia or not.

*CONCLUSION*

Fibroblast growth factor 23 has a key role in FCM induced hypophosphatemia, probably by inducing loss of phosphate in the urine. Short-term clinical impact of hypophosphatemia was not demonstrated.

**Key Words:** Iron deficiency; Hypophosphatemia; Inflammatory bowel disease; Ferric carboxymaltose; Ferric derisomaltose



**Core Tip:** High-dose intravenous iron is an effective treatment for iron deficiency anaemia (IDA) in inflammatory bowel disease (IBD). However, ferric carboxymaltose (FCM) is associated with development of hypophosphatemia. This study of adult IBD patients with IDA investigated the mechanisms and clinical manifestations related to hypophosphatemia after treatment of either FCM or ferric derisomaltose (FDI). The incidence of hypophosphatemia was significantly higher after FCM than FDI, and fibroblast growth factor 23 had a key role, inducing loss of phosphate in the urine along with a significant lowering of 1,25-dihydroxyvitamin D and ionised calcium levels. Short-term clinical impact was not demonstrated.

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## INTRODUCTION

Iron replacement therapy is often needed in patients with inflammatory bowel disease (IBD) because iron deficiency (ID) and ID anaemia (IDA) occur frequently in this patient group[1-3]. A large proportion of IBD patients experience intolerance to oral iron[4]. Additionally, it is asserted that oral iron can lead to an exacerbation of inflammation in the bowel mucosa due to a local effect on the enterocytes[5-7]. Therefore, administration of high-dose iron as an intravenous infusion is an effective, suitable

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and convenient treatment option in IBD. Ferric carboxymaltose (FCM; Ferinject®; Vifor Pharma) and ferric derisomaltose (FDI), previously known as iron isomaltoside (Monofer®; Pharmacosmos A/S), are the most widely used preparations in Europe when high-dose intravenous iron is indicated.

In a recent publication, we described a high incidence of hypophosphatemia in IBD patients who had received treatment with FCM[8]. The mechanism behind the development of hypophosphatemia has been described by Wolf *et al*[9], but probably is not yet fully understood, and has not been investigated in patients with IBD. Fibroblast growth factor 23 (FGF23) is a small peptide hormone, synthesized by osteocytes, which regulates phosphate and vitamin D homeostasis[9]. FGF23 consists of a biologically-active component (full-length, intact FGF23) and inactive C-terminal fragments (C-terminal FGF23). FCM causes an increase in intact FGF23, which triggers the pathophysiological cascade of renal phosphate wasting, suppressed levels of 1,25-dihydroxyvitamin D, and secondary hyperparathyroidism[9]. In contrast, FDI does not appear to induce increased intact FGF23 levels, and is associated with a low incidence of hypophosphatemia[9].

Moderate to severe hypophosphatemia over time, as well as acute severe hypophosphatemia, can lead to serious complications, *e.g.*, respiratory failure, haemolysis, left ventricular failure, and rhabdomyolysis[10-13]. Development of osteomalacia with pseudo-fractures has been found in patients with sustained hypophosphatemia [14-17]. However, there are uncertainties with regard to both the frequency of symptoms and the clinical impact of hypophosphatemia.

Reduced quality of life (QoL) is common and well-documented in IBD patients due to chronic inflammation in the gut and the occurrence of extra-intestinal manifestations[18,19]. Therefore, addressing additional symptoms and implications of hypophosphatemia in this patient group is a challenge, and no specific questionnaire related to hypophosphatemia is available.

In this short-term study, we aimed to investigate the mechanisms causing the development of hypophosphatemia in IBD patients, with ID or IDA, who received one high-dose (1000 mg) infusion of iron. Moreover, we aimed to document symptoms and clinical manifestations related to hypophosphatemia.

## MATERIALS AND METHODS

### *Study design and patient population*

This prospective observational study was conducted between February 1, 2017 and July 1, 2018. The study design and patient recruitment have previously been described in detail (Detlie *et al*[8]). In brief, adult IBD patients (> 18 years) diagnosed with ID or IDA (according to European Crohn's and Colitis Organisation guidelines)[2] were recruited at two separate study sites in the southeast region of Norway and treated with either FCM or FDI.

Eligible patients were prescribed 1000 mg of high-dose intravenous iron, FCM (50 mg/mL) or iron derisomaltose (100 mg/mL), administered as a single dose. Patients who had received high-dose intravenous iron treatment or a packed red blood cell transfusion within 3 mo of study entry, or for whom high-dose intravenous iron treatment was contraindicated, were not included in the study.

Enrolment continued until at least 50 consecutive patients with complete adherence to the study protocol were recruited at each site (a total of more than 100 patients) ( Supplementary Figure 1). The enrolment period was followed by a prospective observation period, which lasted ≤ 7 wk for each patient and included three study visits.

Study inclusion was performed at baseline, at which time intravenous iron treatment was administered. Patients attended the clinic at week 2 (10-15 d) and at week 6 (5-7 wk) following intravenous iron treatment. Each patient could receive only one infusion within an approximate 2-mo period after consenting to study participation.

### *Study assessments and data collection*

Blood analysis at each study visit included ionised calcium, creatinine, phosphate, parathyroid hormone (PTH) and vitamin D (25-hydroxyvitamin D).

Blood samples were also frozen and sent to Medizinische Universität Innsbruck, Universitätsklinik für Innere Medizin I, for analysis of 1,25-dihydroxyvitamin D, intact and C-terminal FGF23. The Kainos FGF-23 ELISA Kit was used for the FGF23 analysis. The assay for intact FGF23 measures only full-length peptide, whereas the assay for C-

terminal FGF23 measures full-length peptide and the C-terminal fragments thereby representing total FGF23.

Spot urine samples were collected at each study visit and analysed for urine phosphate and urine creatinine. A calculation of the fractional excretion of phosphate rate ($FEPO_4$) was then performed using the formula, $FEPO_4$ = (urine phosphate × plasma creatinine × 100)/(plasma phosphate × urine creatinine). Oslo University Hospital Ullevål used the Roche analysis method (Roche/Hitachi Cobas® C systems PHOS2 and CREP2) while Akershus University Hospital used the Vitros analysis (VITROS® MicroSlide Assay 5.1 FS Diluent Pack 3). The slight sensitivity difference between the two analytical methods was minimized by recalculating $FEPO_4$ using the above-mentioned formula.

Symptoms that might be related to hypophosphatemia were assessed at each of the three study visits using the MicroRPM™ (CareFusion) test to determine respiratory muscle function by measuring maximum inspiratory and maximum expiratory pressure, a health-related QoL questionnaire (36-item short form health survey, SF-36), and a visual analogue scale (VAS).

For the MicroRPM™ respiratory function test, patients were asked to inhale and exhale as hard as possible. The test was repeated three times at every visit, and the best result of the three attempts was registered.

The SF-36 is a generic, self-administered questionnaire containing 36 items[20]. The items are divided into eight multi-item scales that reflect general health, physical functioning, role limitations due to physical problems, bodily pain, vitality, mental health, social functioning, and role limitations due to emotional problems. Each scale is transformed into a 1-100 scale, where a lower score represents more disability. The processing of raw SF-36 data into results was executed according to the SF-36 scoring algorithms[21].

The VAS is a 10 cm line on which the patient is asked to place a vertical mark to indicate the level of intensity of a symptom that best fits his or her experience. Scores range from 0-100 (mm) where a higher score represents greater symptom intensity. The VAS was used to assess general weakness, fatigue, joint pain, joint stiffness, muscle pain, bone and skeletal pain, and difficulties performing daily activities.

All demographic information was collected from patients' medical records and was entered into an electronic case report form.

The study was completed when all enrolled patients had received intravenous iron administration, had attended all three study visits, and had fulfilled the requirements of the study protocol.

### Study outcomes

Serum phosphate, PTH, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, ionised calcium, creatinine, intact and C-terminal FGF23, and $FEPO_4$ (urine phosphate and urine creatinine) were measured in order to assess possible mechanisms behind the development of hypophosphatemia after intravenous iron treatment. Results from the FCM treatment group were compared with results from the FDI treatment group.

Hypophosphatemia was defined as a serum phosphate level < 0.8 mmol/L (< 2.5 mg/dL). The clinical impact of hypophosphatemia was evaluated at week 2 and week 6 using the respiratory muscle function test, SF-36, and the VAS score. In relation to the assessment of clinical impact, the hypophosphatemia group was defined as patients experiencing hypophosphatemia at both week 2 and week 6. Results for patients with hypophosphatemia were compared with results for patients without hypophosphatemia, independent of treatment group.

### Statistical analysis

This study was designed to achieve 80% power to detect a difference in the primary outcome, which was the incidence of hypophosphatemia (previously described by Detlie *et al*[8]). Hence, the MicroRPM™ respiratory test, SF-36, and VAS scores were not used to justify sample size.

Data are presented descriptively, as mean with SD or 95% confidence intervals for continuous variables, and as the number of exposed patients (with proportions) for categorical variables. Hypothesis tests for differences in change between treatment groups, change from baseline, and groups with or without hypophosphatemia, were conducted using paired *t*-tests. All analyses were performed in R. A $P$ value of < 0.05 was considered significant.

### Ethical considerations

The study protocol was approved by the relevant local regulatory and ethical

committees and adhered to the applicable laws on data protection. A study registration application was sent to the EudraCT system with the application No. 2016-003476-41, but the application was deemed unnecessary since there were no indications of a medical intervention study.

All patients gave informed consent before inclusion into the study, and the study was performed in accordance with the principles for post-authorisation safety studies, according to Good Clinical Practice guidelines.

All biological material obtained from patients was destroyed after analysis, as were the frozen blood samples sent to the Medical University of Innsbruck.

Study nurses were blinded to the results of laboratory findings but, for safety reasons, the primary investigator at each study centre was not blinded.

## RESULTS

Of the 130 patients screened for this study, 106 patients (52 patients at Oslo University Hospital Ullevål and 54 patients at Akershus University Hospital) were included in the analyses. Demographic and clinical characteristics of the patients have previously been described[8].

Data for serum phosphate, $FEPO_4$, intact and C-terminal FGF23, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, ionised calcium, and PTH at baseline and at each study visit are shown in Table 1. A sub-analysis of the same data, stratified according to hypophosphatemia status (with/without) at week 2 and at week 6, is shown in Table 2.

### *Serum phosphate and urinary excretion of phosphate*

As previously described, following treatment with FCM, hypophosphatemia was present in 72.5% (37/51) of patients at week 2, and in 21.6% (11/51) of patients at week 6. In comparison, in the FDI treatment group, 11.3% (6/53) of patients had hypophosphatemia at week 2, and 3.7% (2/54) at week 6. There were no new incidences of hypophosphatemia at week 6. The difference in incidence was highly significant at both week 2 and 6 ($P < 0.001$ and $P < 0.013$, respectively)[8]. These findings are consistent with the mean urine $FEPO_4$ that was significantly ($P = 0.004$) higher at week 2 in the FCM treatment group compared with the FDI treatment group, and still elevated (though declining) at week 6 in the FCM group (Table 1 and Figure 1A). In the sub-analysis, the FDI-treated patients with hypophosphatemia ($n = 6$) had numerically increased $FEPO_4$ (Table 2).

Patients in both treatment groups without hypophosphatemia at week 2 also experienced an increase in $FEPO_4$ at week 2 compared to baseline values, but urinary phosphate excretion declined again at week 6 in these patients.

### *FGF23*

There was a significant ($P < 0.001$) increase in intact FGF23 from baseline to week 2 after infusion of FCM, compared with the FDI treatment group (Table 1 and Figure 1B). At week 6, intact FGF23 values in the FCM treatment group had returned close to baseline. In comparison, after FDI treatment no such increases were found (Figure 1B). At baseline, the serum concentration of C-terminal FGF23 was higher in the FDI treatment group than in the FCM treatment group (Table 1), and declined after FDI infusion (Figure 1C). This high value at baseline was not seen in the FCM treatment group (Table 1), which is probably compatible with the less severe ID/IDA seen in the FCM group[8].

In the sub-analysis, for the FCM-treated patients with hypophosphatemia, intact FGF23 was significantly increased compared with FCM-treated patients without hypophosphatemia at week 2 and at week 6 (Table 2). In the FCM-treated patients who had normal phosphate, intact FGF23 was not increased at week 2 or week 6.

For FDI-treated patients, the sub-analysis showed that there was no significant difference in mean intact FGF23 Levels between patients with/without hypophosphatemia at week 2 or at week 6 (Table 2). At week 2, only one FDI-treated patient with hypophosphatemia had significantly increased intact FGF23; the other five patients with hypophosphatemia had minimal change in their intact FGF23 values.

### *Vitamin D*

There were no significant differences between the treatment groups in the concentration of 25-hydroxyvitamin D throughout the study period (Figure 1D). However, the sub-analysis showed that 25-hydroxyvitamin D concentrations were lower at week

**Table 1 Descriptive data for laboratory parameters at baseline, at week 2 and at week 6**

| Analysis | High-dose intravenous iron | Baseline, mean ± SD | Week 2, mean ± SD | Difference from baseline at week 2 | Difference between FCM and FDI at week 2 (95%CI) | *P* value | Week 6, mean ± SD | Difference from baseline at week 6 | Difference between FCM and FDI at week 6 (95%CI) | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| Serum phosphate, mmol/L (ref. value 0.8-1.65) | FCM | 1.07 ± 0.2 | 0.65 ± 0.2 | -0.417 | -0.344 (-0.427 to -0.260) | < 0.001 | 1.00 ± 0.3 | -0.072 | -0.070 (-0.144 to 0.004) | 0.064 |
| | FDI | 1.15 ± 0.2 | 1.07 ± 0.2 | -0.073 | | | 1.14 ± 0.2 | -0.002 | | |
| Intact FGF23, pg/mL (ref. value 11.50-48.90) | FCM | 43.42 ± 14.2 | 91.61 ± 63.8 | 49.205 | 45.312 (25.982 to 64.697) | < 0.001 | 44.79 ± 23.1 | 1.718 | 1.559 (-6.407 to 9.525) | 0.698 |
| | FDI | 43.88 ± 14.5 | 47.77 ± 22.1 | 3.892 | | | 44.04 ± 16.6 | 0.159 | | |
| C-terminal FGF23, pmol/L (ref. value 0.30-3.00) | FCM | 2.46 ± 3.2 | 1.68 ± 1.3 | -0.756 | 6.783 (-1.319 to 14.885) | 0.099 | 0.94 ± 1.2 | -1.507 | 5.124 (-1.310 to 11.558) | 0.116 |
| | FDI | 8.87 ± 30.6 | 1.33 ± 1.4 | -7.539 | | | 2.24 ± 7.6 | -6.632 | | |
| $FEPO_4$, % (ref. value N/A) | FCM | 9.95 ± 5.8 | 18.70 ± 10.8 | 9.946 | 5.375 (1.801 to 8.95) | 0.004 | 13.68 ± 11.3 | 4.210 | 3.326 (-0.309 to 6.96) | 0.072 |
| | FDI | 12.55 ± 5.9 | 17.03 ± 8.6 | 4.570 | | | 13.37 ± 6.0 | 0.884 | | |
| PTH, pmol/L (ref. value 1.5-7.0) | FCM | 5.46 ± 2.6 | 7.02 ± 3.4 | 1.608 | 0.442 (-0.561 to 1.445) | 0.384 | 5.97 ± 3.3 | 0.767 | 0.590 (-0.358 to 1.539) | 0.220 |
| | FDI | 5.51 ± 2.6 | 6.72 ± 3.4 | 1.166 | | | 5.69 ± 2.3 | 0.176 | | |
| Ionised calcium, mmol/L (ref. value 1.16-1.32) | FCM | 1.21 ± 0.0 | 1.20 ± 0.0 | -0.015 | -0.020 (-0.035 to -0.004) | 0.012 | 1.21 ± 0.0 | 0.000 | -0.018 (-0.036 to -0.001) | 0.044 |
| | FDI | 1.23 ± 0.0 | 1.23 ± 0.1 | 0.005 | | | 1.25 ± 0.0 | 0.018 | | |
| 25-hydroxyvitamin D, nmol/L (ref. value 50-125) | FCM | 58.35 ± 24.4 | 57.13 ± 23.1 | -1.212 | -2.133 (-6.238 to 1.972) | 0.305 | 57.48 ± 20.8 | -0.865 | -0.160 (-7.078 to 6.759) | 0.964 |
| | FDI | 63.51 ± 21.9 | 64.63 ± 20.0 | 0.922 | | | 62.75 ± 21.1 | -0.706 | | |
| 1,25-dihydroxyvitamin D, ng/L (ref. value 20-79) | FCM | 51.10 ± 19.2 | 28.77 ± 17.9 | -21.074 | -16.463 (-24.487 to -8.438) | < 0.001 | 53.78 ± 20.2 | 3.218 | 5.357 (-3.164 to 13.878) | 0.215 |
| | FDI | 52.85 ± 20.4 | 48.24 ± 17.7 | -4.611 | | | 50.71 ± 19.8 | -2.139 | | |

Normal phosphate levels: > 0.8 mmol/L = > 2.48 mg/dL; Mild hypophosphatemia 0.79-0.6 mmol/L = 2.44-1.86 mg/dL; Moderate hypophosphatemia 0.59-0.32 mmol/L = 1.83-0.99 mg/dL; Severe hypophosphatemia < 0.32 mmol/L = < 0.99 mg/dL. CI: Confidence interval; FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; FEPO4: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone; N/A: Not applicable.

6 in the two FDI-treated patients with hypophosphatemia when compared with baseline concentrations within the same group (Tables 1 and 2). At week 2, 1,25-dihydroxyvitamin D concentrations were significantly lower in patients who received FCM compared with patients who received FDI (Table 1). In the FCM-treatment group, the mean concentration of 1,25-dihydroxyvitamin D returned to baseline at week 6 (Table 1 and Figure 1E). However, the sub-analysis revealed that, for the FCM-treated patients with hypophosphatemia, low 1,25-dihydroxyvitamin D levels persisted at week 6 (Table 2). In the subgroups of patients without hypophosphatemia, 1,25-dihydroxyvitamin D levels were relatively unchanged.

In our cohort, we identified 36 patients (34.0%) with vitamin D deficiency (25-hydroxyvitamin D < 50 nmol/L) at baseline; 10 of these patients had severe vitamin D deficiency (25-hydroxyvitamin D < 30 nmol/L). The distribution of these patients was equal in the two treatment groups, as well as equally distributed across disease states – ulcerative colitis and Crohn's disease. Moreover, we found no association between low levels of vitamin D and development of hypophosphatemia.

**Table 2 Laboratory parameters for patients stratified by hypophosphatemia status (with/without) at week 2 and at week 6**

| Analysis | High-dose intravenous iron | Week 2 serum phosphate ≥ 0.8 mmol/L, mean ± SD | Week 2 serum phosphate < 0.8 mmol/L, mean ± SD | Difference | *P* value | Week 6 serum phosphate ≥ 0.8 mmol/L, mean ± SD | Week 6 serum phosphate < 0.8 mmol/L, mean ± SD | Difference | *P* value |
|---|---|---|---|---|---|---|---|---|---|
| Intact FGF23, pg/mL (ref. value 11.50-48.90) | FCM | 33.71 ± 10.56 (*n* = 14/51) | 114.33 ± 62.22 (*n* = 37/51) | 80.62 | < 0.001 | 39.30 ± 16.79) (*n* = 40/51) | 66.86 ± 30.05 (*n* = 11/51) | 27.56 | 0.013 |
| | FDI | 45.82 ± 16.90 (*n* = 47/53) | 57.16 ± 46.35 (*n* = 6/53) | 11.43 | 0.577 | 43.85 ± 16.83 (*n* = 52/54) | 48.93 ± 10.13 (*n* = 2/54) | 5.08 | 0.604 |
| C-terminal FGF23, pmol/L (ref. value 0.30-3.00) | FCM | 1.03 ± 0.99 (*n* = 14/51) | 1.93 ± 1.33 (*n* = 37/51) | 0.9 | 0.014 | 0.76 ± 0.63 (*n* = 40/51) | 1.64 ± 2.14 (*n* = 11/51) | 0.88 | 0.206 |
| | FDI | 1.36 ± 1.47 (*n* = 47/53) | 1.08 ± 0.72 (*n* = 6/53) | -0.28 | 0.465 | 2.27 ± 7.79 (*n* = 52/54) | 1.31 ± 0.33 (*n* = 2/54) | -0.97 | 0.384 |
| $FEPO_4$, % (ref. value N/A) | FCM | 13.35 ± 5.94 (*n* = 12/46) | 20.59 ± 11.60 (*n* = 34/46) | 7.24 | 0.009 | 9.62 ± 6.56 (*n* = 36/47) | 26.99 ± 13.38 (*n* = 11/47) | 17.37 | 0.001 |
| | FDI | 15.97 ± 7.85 (*n* = 46/52) | 25.11 ± 10.22 (*n* = 6/52) | 9.14 | 0.080 | 13.00 ± 5.79 (*n* = 52/54) | 22.85 ± 4.41 (*n* = 2/54) | 9.85 | 0.176 |
| PTH, pmol/L (ref. value 1.5-7.0) | FCM | 5.46 ± 2.83 (*n* = 14/48) | 7.46 ± 3.23 (*n* = 34/48) | 2 | 0.042 | 5.22 ± 2.39 (*n* = 39/47) | 9.93 ± 4.43) (*n* = 8/47) | 4.71 | 0.020 |
| | FDI | 6.28 ± 2.97 (*n* = 47/53) | 10.13 ± 4.70 (*n* = 6/53) | 3.85 | 0.102 | 5.66 ± 2.32 (*n* = 52/54) | 6.40 ± 1.70 (*n* = 2/54) | 0.74 | 0.649 |
| Ionised calcium, mmol/L (ref. value 1.16-1.32) | FCM | 1.21 ± 0.32 (*n* = 13/50) | 1.19 ± 0.05 (*n* = 37/50) | -0.02 | 0.336 | 1.21 ± 0.05 (*n* = 40/51) | 1.22 ± 0.04 (*n* = 11/51) | 0.01 | 0.496 |
| | FDI | 1.24 ± 0.45 (*n* = 46/52) | 1.21 ± 0.09 (*n* = 6/52) | -0.03 | 0.422 | 1.25 ± 0.05 (*n* = 51/53) | 1.23 ± 0.02 (*n* = 2/53) | -0.02 | 0.314 |
| 25-hydroxyvitamin D, nmol/L (ref. value 50-125) | FCM | 63.00 ± 30.94 (*n* = 14/51) | 54.54 ± 19.69 (*n* = 37/51) | -8.46 | 0.354 | 60.05 ± 21.68 (*n* = 40/51) | 45.45 ± 10.00 (*n* = 11/51) | -14.6 | 0.002 |
| | FDI | 64.16 ± 20.30 (*n* = 45/51) | 64.83 ± 18.93 (*n* = 6/51) | 0.67 | 0.937 | 63.68 ± 20.97 (*n* = 50/52) | 39.50 ± 2.12 (*n* = 2/52) | -24.18 | < 0.001 |
| 1,25-dihydroxyvitamin D, ng/L (ref. value 20-79) | FCM | 46.34 ± 19.10 (*n* = 14/50) | 21.46 ± 11.63 (*n* = 36/50) | -24.88 | < 0.001 | 56.76 ± 20.27 (*n* = 40/50) | 40.89 ± 15.95 (*n* = 10/50) | -15.87 | 0.016 |
| | FDI | 49.05 ± 18.52 (*n* = 47/53) | 45.72 ± 7.15 (*n* = 6/53) | -3.33 | 0.414 | 50.88 ± 19.92 (*n* = 52/54) | 46.45 ± 21.85 (*n* = 2/54) | -4.43 | 0.822 |

Normal phosphate levels: > 0.8 mmol/L = > 2.48 mg/dL; Mild hypophosphatemia 0.79-0.6 mmol/L = 2.44-1.86 mg/dL; Moderate hypophosphatemia 0.59-0.32 mmol/L = 1.83-0.99 mg/dL; Severe hypophosphatemia < 0.32 mmol/L = < 0.99 mg/dL. FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; FEPO4: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone; N/A: Not applicable.

### *Calcium and PTH*

Ionised calcium values dropped significantly from baseline to week 2 in the FCM treatment group compared with the FDI treatment group ($P < 0.012$) but stayed within normal range. The mean values in the FCM group had increased by week 6, but the between-group difference was still significant ($P < 0.044$). Calcium values remained stable throughout the study in the FDI treatment group (Figure 1F), and in the subgroup of FCM-treated patients who did not develop hypophosphatemia. The sub-analysis showed that there was a numerically lower level of ionised calcium in the FDI-treated patients with hypophosphatemia than in the FDI-treated patients without hypophosphatemia (Table 2).

PTH values were elevated (> 7 pmol/L) in 28 patients (26.4%) at baseline; the distribution was similar between treatment groups. PTH concentrations were similar between treatment groups at baseline, and no significant between-group differences were observed in mean PTH concentrations at week 2, and at week 6 (Table 1). PTH values increased in both treatment groups at week 2 and decreased again at week 6 (Figure 1G). The sub-analysis indicated that the increase in PTH in both treatment groups was mainly driven by the patients who developed hypophosphatemia, with significant differences at week 2 and week 6 for the FCM-treated patients with hypophosphatemia compared to FCM-treated patients without hypophosphatemia (Table 2).

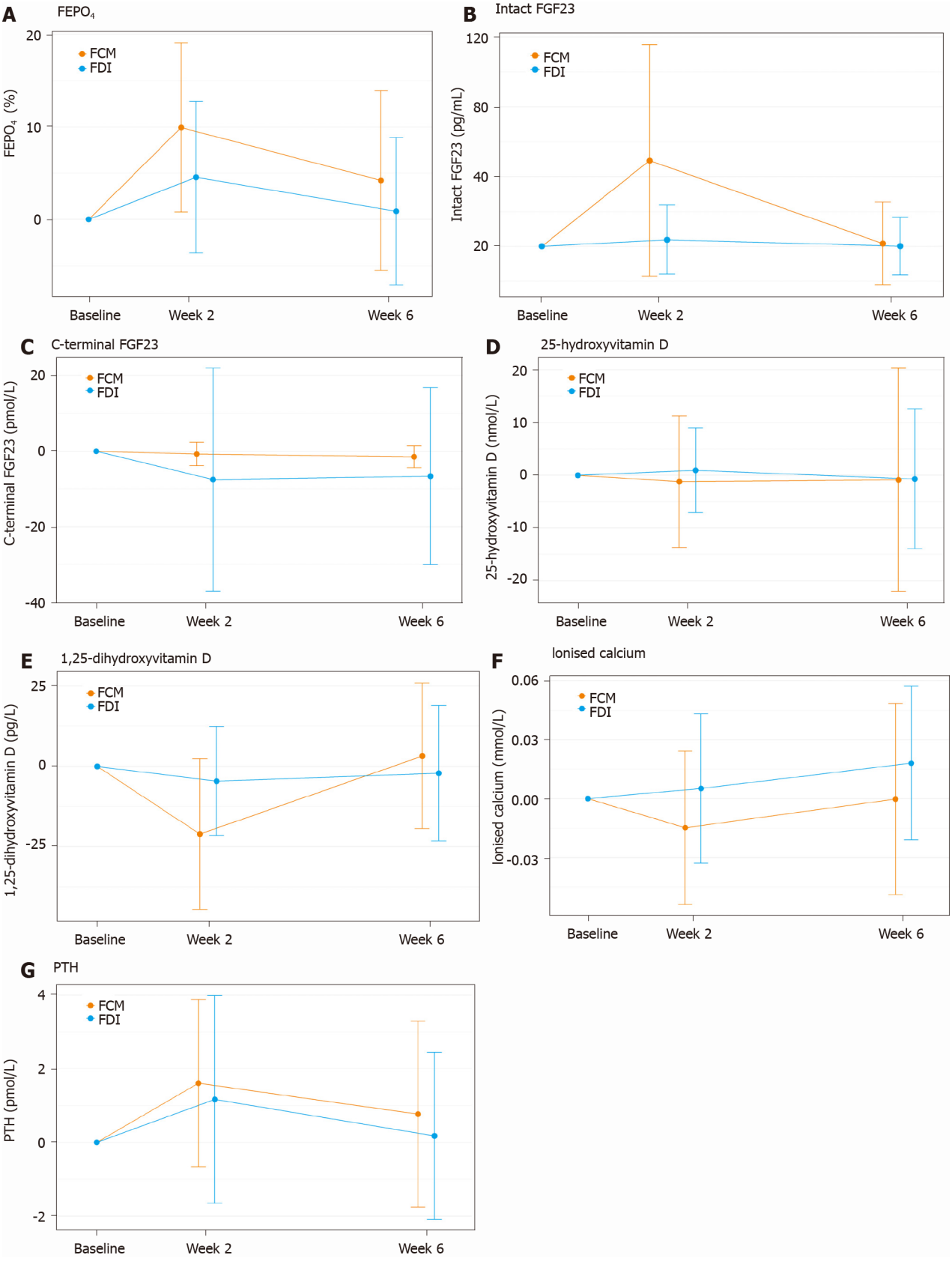


**Figure 1 mean ± SD change from baseline in laboratory parameters in inflammatory bowel disease patients with iron deficiency/iron deficiency anaemia treated with a single 1000 mg intravenous dose of ferric carboxymaltose or ferric derisomaltose.** A: Fractional excretion of phosphate; B: Intact fibroblast growth factor 23; C: C-terminal fibroblast growth factor 23; D: 25-Hydroxyvitamin D; E: 1,25-Hydroxyvitamin D; F: Ionised calcium; G: Parathyroid hormone. FCM: Ferric carboxymaltose; FDI: Ferric derisomaltose; $FEPO_4$: Fractional excretion of phosphate; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone.

### Respiratory muscle function tests

In the comparison of patients who developed hypophosphatemia *vs* those who did not develop hypophosphatemia, independent of treatment group, no significant differences were observed in the respiratory muscle function test results. The differences between patients with hypophosphatemia and those with normal phosphate values were minimal and the standard deviation was wide in both groups (Figure 2).

### SF-36

The results of the SF-36 QoL assessment are presented in Table 3. Overall, there were no significant differences between patient groups with or without hypophosphatemia at baseline and at any time point during the study. The mean scores at baseline in both treatment groups were generally low.

### VAS scores

There were no significant differences in VAS scores between the groups of patients with/without hypophosphatemia at week 2 and at week 6 (Table 4). Overall, VAS scores were elevated at baseline. However, the group of patients who developed hypophosphatemia had lower VAS scores at baseline for the items joint pain, muscle pain, and bone and skeletal pain, compared to the group of patients who did not develop hypophosphatemia; between-group differences were not significant for these items. There was, however, a significant between-group difference ($P < 0.001$) at baseline for the VAS joint stiffness item score, with lower values in the group of patients who developed hypophosphatemia.

## DISCUSSION

Our study indicates that FGF23 plays an important role in the development of hypophosphatemia in IBD patients treated with FCM. In these patients, a high level of intact FGF23, an increased excretion of phosphate in the urine, a decrease of 1,25-dihydroxyvitamin D and of serum calcium levels, and a slight elevation of PTH, was demonstrated.

Previous clinical trials of FCM have shown similar results[9,22]. However, for the most part, these studies have been conducted in healthy and, predominantly, female populations. The role of FGF23 has also been described in earlier publications[23-26]. Regulation of phosphate concentrations in the body seems to be strongly influenced by intact FGF23, which reduces phosphate reabsorption in the proximal tubules in the kidneys and inhibits production of 1,25-dihydroxyvitamin D, probably by inhibiting the activity of the enzyme 25-hydroxyvitamin D-1a-hydroxylase and increased expression of 24-hydroxylase[24,26]. Our findings suggest that FCM could have a direct impact on cleavage of FGF23, resulting in a high level of intact FGF23 and consequent phosphate wasting. This might also explain why baseline phosphate level does not predict the development of mild or severe hypophosphatemia, due to the inappropriate excretion of available phosphate in the urine, following FCM treatment[8]. We also observed a decrease in 1,25-dihydroxyvitamin D (the active vitamin D metabolite), a decrease in ionised calcium, and development of secondary hyperparathyroidism. This might explain why some patients treated with FCM still had hypophosphatemia six weeks after treatment, when the intact FGF23 values had normalized (Table 2) since elevated PTH promotes excretion of phosphate in the urine[9,27,28].

The majority of patients in the FCM treatment group developed hypophosphatemia at week 2. The remaining patients did not develop hypophosphatemia and had unchanged levels of intact FGF23. So, there is a clear association between the development of high levels of intact FGF23 and hypophosphatemia. Therefore, it can only be speculated that there might be some individual factors related to the handling of FCM that cause the majority of patients treated with FCM to develop hypophosphatemia, whereas others do not. Neither is it known if any individual patient would develop hypophosphatemia on subsequent administrations of FCM, or if the effect of FCM treatment on phosphate wasting is indiscriminate. Perhaps some patients are protected against the influence of FCM on the enzyme responsible for FGF23 protein cleavage. From our results, we postulate that the mechanism of FCM-induced hypophosphatemia is not related to IBD; instead, it appears to be independently connected to the drug itself.

**Table 3 Descriptive 36-item short form health survey scores for patient groups with/without hypophosphatemia independent of treatment group**

| SF-36 scale item | Normal phosphate population, baseline, mean ± SD | Hypophosphatemia population, baseline, mean ± SD | Difference[1] at baseline, mean (95%CI) | Normal phosphate population, change at week 2, mean ± SD | Hypophosphatemia population, change at week 2, mean ± SD | Difference[1] at week 2, mean (95%CI) | Normal phosphate population, change at week 6, mean ± SD | Hypophosphatemia population, change at week 6, mean ± SD | Difference[1] at week 6, mean (95%CI) |
|---|---|---|---|---|---|---|---|---|---|
| General health | 50.91 ± 20.1 | 48.50 ± 26.1 | 2.4 (-14.5 to 19.4) | -0.45 ± 12.9 | 0.75 ± 12.2 | -1.2 (-9.2 to 6.8) | 3.06 ± 16.3 | 3.25 ± 15.1 | -0.2 (-10.2 to 9.8) |
| Physical functioning | 78.72 ± 22.4 | 86.11 ± 21.1 | -7.4 (-21.3 to 6.6) | 2.63 ± 10.9 | -0.28 ± 6.3 | 2.9 (-1.6 to 7.4) | 3.97 ± 14.5 | -0.28 ± 4.7 | 4.2 (0.2 to 8.3) |
| Role limitations due to physical problems | 47.61 ± 42.8 | 66.67 ± 40.4 | -19.1 (-45.7 to 7.6) | -4.08 ± 28.1 | -6.25 ± 18.8 | 2.2 (-10.8 to 15.1) | 11.32 ± 39.2 | 2.08 ± 16.7 | 9.2 (-3.7 to 22.1) |
| Bodily pain | 65.65 ± 25.1 | 67.17 ± 27.6 | -1.5 (-19.6 to 16.5) | 0.68 ± 18.9 | 4.42 ± 12.2 | -3.7 (-12.2 to 4.7) | 3.40 ± 19.8 | 10.83 ± 19.1 | -7.4 (-20.0 to 5.2) |
| Vitality | 37.98 ± 22.0 | 40.00 ± 22.4 | -2.0 (-16.7 to 12.7) | 4.05 ± 13.1 | 7.50 ± 15.9 | -3.4 (-13.8 to 6.9) | 10.89 ± 18.0 | 12.92 ± 16.0 | -2.0 (-12.7 to 8.6) |
| Mental health | 71.72 ± 18.9 | 70.33 ± 16.9 | 1.4 (-9.9 to 12.6) | 3.37 ± 9.0 | 4.33 ± 5.0 | -1.0 (-4.5 to 2.6) | 4.89 ± 13.2 | 1.67 ± 11.1 | 3.2 (-4.2 to 10.7) |
| Social functioning | 68.35 ± 26.3 | 68.75 ± 30.4 | -0.4 (-20.2 to 19.4) | 3.86 ± 16.7 | 7.29 ± 13.5 | -3.4 (-12.5 to 5.6) | 8.70 ± 20.7 | 7.29 ± 15.5 | 1.4 (-9.1 to 11.9) |
| Role limitations due to emotional problems | 67.91 ± 41.5 | 72.22 ± 42.2 | -4.3 (-32.1 to 23.4) | -2.66 ± 34.8 | -8.33 ± 37.9 | 5.7 (-19.1 to 30.5) | 6.52 ± 38.0 | -2.78 ± 26.4 | 9.3 (-8.8 to 27.4) |

Hypophosphatemia defined as serum phosphate < 0.8 mmol/L (< 2.5 mg/dL).

[1]Differences are normal phosphate group minus hypophosphatemia group. CI: Confidence interval; SF-36: 36-Item short form health survey.

A few patients who received treatment with FDI also developed hypophosphatemia but, unlike those receiving FCM, these patients did not on average have significantly elevated intact FGF23 Levels when assessed at week 2, which would suggest a different underlying mechanism. A transient increase in intact FGF23 during the first 2 wk in patients experiencing hypophosphatemia cannot be ruled out, as data were not collected during this time period. A numerical increase in PTH was observed at week 2 along with decreased ionised calcium, and decreased 25-hydroxyvitamin D at week 6. It is not clear whether these observations are the result of a transient increase of intact FGF23 during the first 2 wk, or solely a physiological response to a rapid correction of ID, or simply an artefact due to the low numbers of FDI patients who developed hypophosphatemia. The general physiological response of mineral metabolism markers to rapid ID correction is not fully elucidated and is an area of further research.

**Table 4 Descriptive visual analogue scale score for patient groups with/without hypophosphatemia independent of treatment group**

| VAS item | Normal phosphate group, baseline, mean ± SD | Hypophosphatemia group, baseline, mean ± SD | Difference[1] at baseline, mean (95%CI) | *P* value | Normal phosphate group, change at week 2, mean ± SD | Hypophosphatemia group, change at week 2, mean ± SD | Difference[1] at week 2, mean (95%CI) | *P* value | Normal phosphate group, change at week 6, mean ± SD | Hypophosphatemia group, change at week 6, mean ± SD | Difference[1] at week 6, mean (95%CI) | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| General weakness | 43.54 ± 28.7 | 31.17 ± 30.5 | 12.4 (-7.6 to 32.4) | 0.205 | -4.98 ± 14.9 | -1.50 ± 17.2 | -3.5 (-14.7 to 7.7) | 0.515 | -10.64 ± 21.9) | -6.33 ± 25.7 | -4.3 (-21.1 to 12.5) | 0.589 |
| Fatigue | 37.53 ± 30.0 | 29.58 ± 30.1 | 7.9 (-11.8 to 27.7) | 0.403 | -3.31 ± 19.4 | -1.33 ± 17.9 | -2.0 (-13.9 to 9.9) | 0.728 | -8.93 ± 22.0 | -5.17 ± 26.7 | -3.8 (-21.2 to 13.6) | 0.648 |
| Joint pain | 17.30 ± 23.3 | 8.25 ± 14.8 | 9.1 (-1.2 to 19.3) | 0.081 | 0.32 ± 14.3 | -3.09 ± 6.9 | 3.4 (-1.9 to 8.7) | 0.197 | 2.17 ± 18.0 | 0.17 ± 7.4 | 2.0 (-3.8 to 7.8) | 0..489 |
| Joint stiffness | 13.31 ± 22.3 | 1.42 ± 3.4 | 11.9 (6.9 to 16.8) | < 0.001 | -0.57 ± 18.1 | -0.64 (2.1) | 0.1 (-3.9 to 4.0) | 0.972 | 0.99 ± 17.7 | 1.75 ± 8.5 | -0.8 (-7.1 to 5.6) | 0.807 |
| Muscle pain | 15.35 ± 23.1 | 5.83 ± 14.5 | 9.5 (-0.5 to 19.6) | 0.062 | -1.71 ± 14.6 | 2.45 (8.9) | -4.2 (-10.7 to 2.3) | 0.194 | -0.50 ± 20.6 | -0.17 ± 11.0 | -0.3 (-8.3 to 7.6) | 0.932 |
| Bone and skeletal pain | 7.24 ± 19.4 | 1.92 ± 6.6 | 5.3 (-0.3 to 10.9) | 0.061 | 1.85 ± 20.2 | 2.64 (8.7) | -0.8 (-7.7 to 6.1) | 0.817 | 3.42 ± 18.7 | -1.67 ± 6.8 | 5.1 (-0.5 to 10.7) | 0.076 |
| Difficulties performing daily activities | 34.38 ± 28.5 | 29.67 ± 29.7 | 4.7 (-14.8 to 24.2) | 0.611 | -3.80 ± 14.9 | -2.17 (23.5) | -1.6 (-16.8 to 13.5) | 0.819 | -9.45 ± 20.0 | -11.00 ± 24.8 | 1.6 (-14.6 to 17.7) | 0.839 |

Hypophosphatemia defined as serum phosphate < 0.8 mmol/L (< 2.5 mg/dL).

[1]Differences are normal phosphate group minus hypophosphatemia group. CI: Confidence interval; VAS: Visual analogue scale.

An important observation is that 34% of the study population was vitamin D deficient at baseline with 25-hydroxyvitamin D values < 50 nmol/L and, perhaps more interestingly, 24% of the patients had PTH values compatible with secondary hyperparathyroidism. These findings were equally distributed between the two treatment groups. This disturbance in vitamin D metabolism is unlikely to be a consequence of previous iron infusions since no patients received high-dose intravenous iron treatment during the 6 mo prior to inclusion in this study. The high prevalence of vitamin D deficiency at baseline is in agreement with previous studies of patients with IBD[29]. However, it is important to note that, in our study, many of the samples were taken during the winter months when sun exposure is reduced in Norway, and individuals could therefore be expected to be somewhat vitamin D deficient during this time. Nevertheless, this finding is important since both hypophosphatemia and vitamin D deficiency can contribute to the development of metabolic bone disease, including osteomalacia.

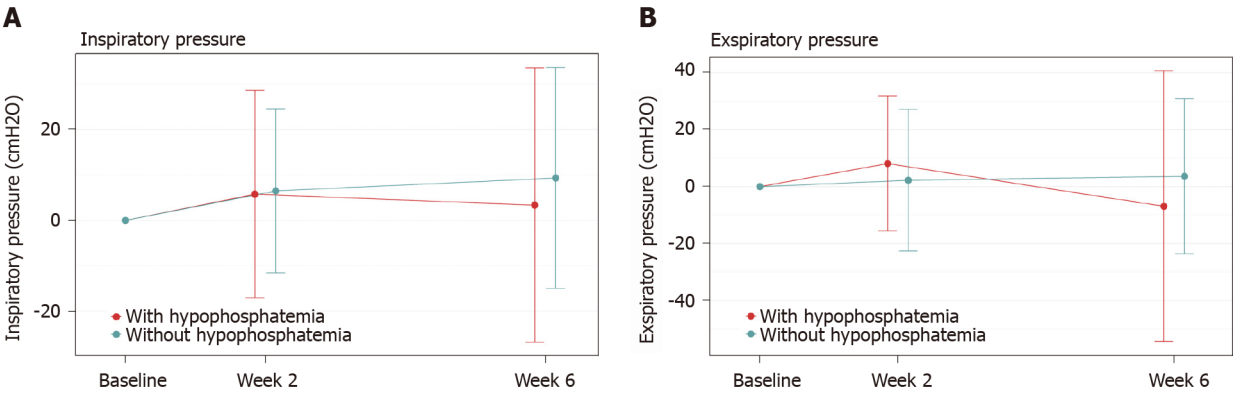


**Figure 2 mean ± SD changes from baseline in respiratory pressure in inflammatory bowel disease patients with iron deficiency/iron deficiency anaemia treated with a single 1000 mg intravenous dose of ferric carboxymaltose or ferric derisomaltose.** A: Inspiratory pressure; B: Expiratory pressure.

Guidelines regarding hypophosphatemia diagnosis, treatment, and follow-up are available, but the possible risk or incidence rate of developing hypophosphatemia with symptoms or complications are rarely mentioned[30]. A risk of developing respiratory failure, rhabdomyolysis, and left ventricular failure due to severe hypophosphatemia has been reported in case series[10]. More recent data also predict an increased risk of developing osteomalacia, especially in long-standing hypophosphatemia[14,15]. What is less well known is the number of patients developing more subtle, but identifiable, symptoms related to hypophosphatemia that are experienced as troublesome and might influence QoL.

With respect to the clinical impact of hypophosphatemia, measuring forced inspiratory and expiratory respiratory pressure can be used as a proxy to assess the physical effect of hypophosphatemia on skeletal and proximal muscles. There are no specific questionnaires available to evaluate the clinical impact of hypophosphatemia. The SF-36 is, however, one of the most commonly applied QoL questionnaires used world-wide in health surveys. Additionally, the VAS score can be used as a general assessment of impact of symptoms, such as fatigue, general weakness, bone and skeletal pain, and joint and muscle conditions. In our study, these three methods were applied to assess clinical impact in patients who developed hypophosphatemia compared to those who did not develop hypophosphatemia. All three methods failed to demonstrate significant differences in clinical impact following one administration of high-dose intravenous iron in this short-term study.

We hypothesize several reasons that might explain these results. In addition to the fact that a type II error cannot be excluded, it can be speculated that the positive effect of the correction of ID or IDA plays a more important role than any short-term negative clinical impact of hypophosphatemia and, hence, the effects of hypophosphatemia would be difficult to discern in our study. Another challenge is that IBD, ID, IDA and hypophosphatemia are associated with similar symptoms and, possibly, similar impacts on daily life. Indeed, assessing the specific impact of hypophosphatemia with questionnaires would, therefore, prove difficult. Since the SF-36 and the VAS questionnaires are not disease- or population-specific, there may be uncertainty surrounding the reliability of the results. Additionally, the patient cohort had more than one dynamic medical condition, with overlapping symptoms, and patients were observed in a longitudinal manner. Certainly, it would be almost impossible to determine which disease state or co-morbidity is reflecting improvement or worsening of clinical status.

The already affected baseline recordings in SF-36 and the VAS score should not go unnoticed. These findings mirror previous studies of IBD populations[31], and reflect the reduced QoL and the intensity of symptoms that these patients experience in general. Finally, the fact that we did not detect clinical consequences in patients who developed hypophosphatemia suggests that, in order to detect overt symptoms and complications, the population size needs to be larger than our sample, as one might expect such complications to be relatively rare. Hence, this needs to be taken into account when considering the expectation of finding significant changes in the clinical outcomes in this study.

## CONCLUSION

In summary, our study has implicated the small peptide hormone FGF23 in the development of hypophosphatemia in IBD patients treated with FCM. An increase in intact FGF23 occurs, which probably results in phosphate wasting in the urine. Assessment of symptoms did not exclude, nor did they demonstrate, any short-term clinical impact of hypophosphatemia in IBD patients treated for ID or IDA with high-dose intravenous iron.

## ARTICLE HIGHLIGHTS

### *Research background*

High-dose intravenous iron is an effective and frequently used treatment option for iron deficiency (ID) or ID anaemia (IDA) in inflammatory bowel disease (IBD). However, treatment with ferric carboxymaltose (FCM) has been associated with the development of hypophosphatemia.

### *Research motivation*

We aimed to investigate the occurrence of hypophosphatemia after treatment with either FCM and ferric derisomaltose (FDI) for ID or IDA in patients with IBD.

### *Research objectives*

In this part of the study, we aimed to disclose underlying mechanism behind the development of hypophosphatemia after treatment with high dose intravenous iron and whether hypophosphatemia had a clinical impact on these patients.

### *Research methods*

A prospective observational study of adult IBD patients with ID or IDA was conducted between February 1, 2017 and July 1, 2018 at two separate university hospitals in the southeast region of Norway. Patients were recruited consecutively and received one dose of 1000 mg of either FCM or FDI, and were followed for an observation period of at least 7 wk at three timepoints; baseline, week 2 and week 6. Blood and urine samples were collected for relevant analyses at all three visits in addition to assessment of clinical symptoms using a respiratory function test, a visual analogue scale, and a health-related quality of life questionnaire.

### *Research results*

Our study results demonstrate an association between FCM treatment and the development of hypophosphatemia by increasing the level of intact Fibroblast Growth Factor 23 (iFGF23) and phosphate wasting in the urine. Moreover, we observed a significant decline in active Vitamin D and ionised calcium. No clinical impact was detected by applying Short Form-36 questionnaire, visual analogue scale score and real-time position management breathing test in an observation period of 6 wk.

### *Research conclusions*

FCM treatment is associated with the development of hypophosphatemia in patients with IBD. This is due to increased formation of iFGF23 which in turn probably results in an increase of urinary phosphate output. No clinical impact was detected nor excluded. Assumably our study is underpowered together with a too short observation period to provide solid information with regard to clinical impact of hypophosphatemia.

### *Research perspectives*

Based on our results we encourage clinicians to be aware of the risk of developing hypophosphatemia after treatment with FCM. Larger studies with a longer observation period to detect possible clinical impact of hypophosphatemia is desirable.

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