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EDITORIAL

Liquid biopsy in colorectal cancer: No longer young, but not yet old

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Abstract

Colorectal cancer (CRC) is one of the most prevalent cancers and the second leading cause of cancer-related deaths worldwide. The treatment strategy employed in CRC patients is becoming highly dependent on molecular characteristics present at diagnosis and during treatment. Liquid biopsy is an emerging field in the management of this cancer, and its relevance as a potential diagnostic, prognostic, monitoring, and therapeutic tool makes it a viable strategy in the clinical management of CRC patients. Liquid biopsy also has certain limitations, but these limitations seem to be at the reach of near-future technological development. In this letter, we focus on the clinical perspectives of liquid biopsy in CRC with particular regard to the various biomarkers recently identified that have been shown to be potentially useful in multiple aspects of early stage or metastatic CRC.

Key Words: Colorectal cancer; Liquid biopsy; Circulating tumor DNA; Diagnosis; Prognosis; Targeted therapy

Core Tip: Liquid biopsy through analysis of biological components, such as circulating nuclear acids, circulating tumor cells, and more recently exosomes in body fluids, has shown good capacity to overcome several limitations faced by conventional tissue biopsies, in particular invasiveness and unrepeatability. Liquid biopsy has shown significant results in clinical applications in different types of cancer, especially colorectal cancer (CRC). Indeed, liquid biopsy can be used to detect CRC at an early stage, make treatment decisions, monitor response to treatment, predict relapses and metastases, reveal tumor heterogeneity, and detect minimal residual disease.

INTRODUCTION

The strategy employed for the treatment of colorectal cancer (CRC) patients is becoming highly dependent on molecular characteristics at diagnosis and during the course of treatment. New therapeutic options have been shown to be effective for metastatic disease, including immune checkpoint inhibitors (ICIs), HER2-directed antibody-drug conjugates, chemotherapy-free regimens for BRAFV600-mutated tumors, and new agents targeting the RAS signaling pathway[1,2].

In recent years, peripheral blood has been extensively studied as a new source of information and alternative to tumor biopsy samples, but its potential has not yet been elucidated. Among the most studied components, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and, more recently, exosomes have been considered promising biomarkers for monitoring treatment response, longitudinal molecular profiling, prognostication, and detection of minimal residual disease[3].

These advances coincided with the progressive increase in the accessibility and cost-effectiveness of next-generation sequencing (NGS) and with the development of new therapeutic approaches, including tumor-agnostic treatments[4].

LIQUID BIOPSY APPLICATION

CTC detection and enumeration have been studied as prognostic markers in several cancers. Sastre *et al* [5] evaluated the prognostic effect of the presence of CTCs detected using the CellSearch System in CRC patients (n = 97). The authors showed an association between CTC detection and stage. With a mean number of 3.4/7.5 mL CTCs, a higher rate of CTC positivity was observed in patients with stage IV disease (60.7%) than in those with stage II-III disease (20.7%-24.1%). Interestingly, no CTCs were detected in the healthy population[5]. Although CTC determination could represent a reliable surrogate for tumor burden, the possibility of longitudinal profiling of the disease offers far greater advantages for clinical practice than mere enumeration.

One of the most promising applications of liquid biopsy is the detection of early-stage CRC. For this purpose, CTC detection and quantification were studied with conflicting results regarding the discrimination of cancerous, precancerous, and other benign lesions[6]. Then, epigenetic changes were investigated to achieve a greater specificity. Methylated SEPT9 promoter DNA has been shown to be a potential biomarker with some limitations[7]. The interpretation of the results was, indeed, highly dependent on the choice of a favorable balance between sensitivity and specificity[8]. The detection of methylated SEPT9 promoter DNA through a real-time polymerase chain reaction assay was validated in a prospective study and recently received FDA approval for CRC screening[9]. However, several limitations deserve to be considered, including the rates of false-positive and negative results, reproducibility, need for confirmatory tests, and schedule of tests over time.

In the last few years, ctDNA has been extensively studied to identify minimal residual disease. A prospective multicenter study conducted by Henriksen *et al*[10] evaluated the preoperative and postoperative ctDNA status in stage I-III CRC. Overall, postoperative ctDNA was associated with relapse-free survival (P < 0.001). In addition, the detection of ctDNA made it possible to anticipate radiological relapse by approximately eight months[10]. Consistent with this finding, Kasi *et al*[11] evaluated the ctDNA status in a cohort of 250 patients using Signatera liquid biopsy. The ctDNA detection rate was significantly associated with stage and response to treatment[11].

Another application of liquid biopsy for the treatment of metastatic CRC involves the monitoring of RAS mutational status to select patients for anti-EGFR rechallenge. In the CRICKET trial, no responses were observed with cetuximab-based chemotherapy in RAS and RAF wild-type mCRC patients who had RAS-mutated ctDNA at the time of progression. Given that RAS mutations in ctDNA could be
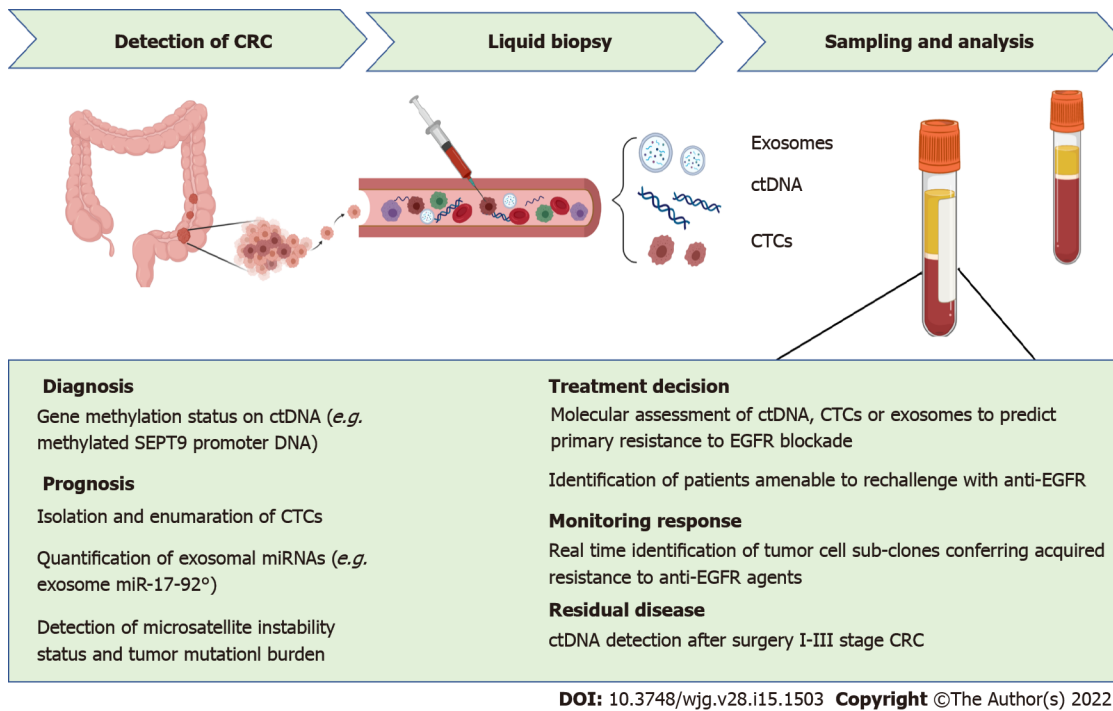
Figure 1 Potential clinical uses of liquid biopsy in colorectal cancer. ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; CRC: Colorectal cancer.

detected in approximately half of the patients at the beginning of third-line treatment[12], longitudinal monitoring of the disease is essential to offer potentially effective treatments to patients with few residual therapeutic opportunities. In addition, molecular selection in chemorefractory patients allows us to avoid unnecessary side effects for ineffective treatments. However, at the time of diagnosis, the molecular alterations in tissue biopsy cannot be replaced with those in liquid biopsy until the key issue of the concordance of RAS mutational status between plasma and tumor tissue is completely resolved. The results may differ depending on the methods used. To date, several prospective trials have demonstrated a high concordance between the two techniques that is approximately equal to 85%-95% [13,14].

Exosome quantification was initially studied as a prognostic factor associated with clinical and pathological parameters and, consequently, survival outcomes[15]. Then, several studies analyzed specific exosomal miRNAs. Among these, Matsumura *et al*[16] showed that CRC patients had higher levels of exosomal miR-17-92a than the control group. In addition, the authors identified the expression of exosomal miR-17-92a in peripheral blood as a prognostic factor for CRC patients[16]. Additionally, exosomal RNAs are used in the early diagnosis of cancers. For example, a panel consisting of two mRNAs (KRTAP5-4 and MAGEA3) and one lncRNA (BCAR4) serves as a promising candidate for CRC diagnosis[17-19].

Other studies are exploring the possibility of detecting other predictors of response, including microsatellite instability status and tumor mutational burden. The noninvasive detection of biomarkers predictive of response to ICIs is eagerly awaited to personalize treatments. However, their identification is profoundly limited by the amount of ctDNA and validation of specific assays. To date, there are no data to support their routine use[20,21].

CONCLUSION

In conclusion, liquid biopsy is no longer considered a mere surrogate of tumor biopsy with minimal invasiveness (Figure 1). New perspectives could radically change clinical practice[22]. Further advances will include refining serum sequencing techniques to gain a deeper understanding of tumor temporal heterogeneity and promote accessibility to tumor-agnostic treatments. In addition, the real-time monitoring of drug resistance beyond RAS may offer the opportunity to guide and monitor new therapies, such as anti-RAS agents, BRAFV600-directed or HER2-directed treatments, and ICIs. In addition, monitoring plasma with amplicon-based NGS in CRC patients may offer high sensitivity in detecting low-frequency mutations and promote the identification of clones with potentially targetable alterations. All these aspects will be crucial to ensure the paradigm of a continuum of care for metastatic CRC patients.

FOOTNOTES

REFERENCES

1   **Xie YH**, Chen YX, Fang JY. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct Target Ther* 2020; **5**: 22 [PMID: 32296018 DOI: 10.1038/s41392-020-0116-z]

2   **Roviello G**, D'Angelo A, Petrioli R, Roviello F, Cianchi F, Nobili S, Mini E, Lavacchi D. Encorafenib, Binimetinib, and Cetuximab in BRAF V600E-Mutated Colorectal Cancer. *Transl Oncol* 2020; **13**: 100795 [PMID: 32470910 DOI: 10.1016/j.tranon.2020.100795]

3   **Ignatiadis M**, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic - implementation issues and future challenges. *Nat Rev Clin Oncol* 2021; **18**: 297-312 [PMID: 33473219 DOI: 10.1038/s41571-020-00457-x]

4   **Lavacchi D**, Roviello G, D'Angelo A. Tumor-Agnostic Treatment for Cancer: When How is Better than Where. *Clin Drug Investig* 2020; **40**: 519-527 [PMID: 32307639 DOI: 10.1007/s40261-020-00915-5]

5   **Sastre J**, Maestro ML, Puente J, Veganzones S, Alfonso R, Rafael S, García-Saenz JA, Vidaurreta M, Martín M, Arroyo M, Sanz-Casla MT, Díaz-Rubio E. Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. *Ann Oncol* 2008; **19**: 935-938 [PMID: 18212090 DOI: 10.1093/annonc/mdm583]

6   **Parkinson DR**, Dracopoli N, Petty BG, Compton C, Cristofanilli M, Deisseroth A, Hayes DF, Kapke G, Kumar P, Lee JSh, Liu MC, McCormack R, Mikulski S, Nagahara L, Pantel K, Pearson-White S, Punnoose EA, Roadcap LT, Schade AE, Scher HI, Sigman CC, Kelloff GJ. Considerations in the development of circulating tumor cell technology for clinical use. *J Transl Med* 2012; **10**: 138 [PMID: 22747748 DOI: 10.1186/1479-5876-10-138]

7   **Sun G**, Meng J, Duan H, Zhang D, Tang Y. Diagnostic Assessment of septin9 DNA Methylation for Colorectal Cancer Using Blood Detection: A Meta-Analysis. *Pathol Oncol Res* 2019; **25**: 1525-1534 [PMID: 30488278 DOI: 10.1007/s12253-018-0559-5]

8   **deVos T**, Tetzner R, Model F, Weiss G, Schuster M, Distler J, Steiger KV, Grützmann R, Pilarsky C, Habermann JK, Fleshner PR, Oubre BM, Day R, Sledziewski AZ, Lofton-Day C. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem* 2009; **55**: 1337-1346 [PMID: 19406918 DOI: 10.1373/clinchem.2008.115808]

9   **Potter NT**, Hurban P, White MN, Whitlock KD, Lofton-Day CE, Tetzner R, Koenig T, Quigley NB, Weiss G. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. *Clin Chem* 2014; **60**: 1183-1191 [PMID: 24938752 DOI: 10.1373/clinchem.2013.221044]

10   **Henriksen TV**, Tarazona N, Frydendahl A, Reinert T, Gimeno-Valiente F, Carbonell-Asins JA, Sharma S, Renner D, Hafez D, Roda D, Huerta M, Roselló S, Madsen AH, Løve US, Andersen PV, Thorlacius-Ussing O, Iversen LH, Gotschalck KA, Sethi H, Aleshin A, Cervantes A, Andersen CL. Circulating Tumor DNA in Stage III Colorectal Cancer, beyond Minimal Residual Disease Detection, toward Assessment of Adjuvant Therapy Efficacy and Clinical Behavior of Recurrences. *Clin Cancer Res* 2022; **28**: 507-517 [PMID: 34625408 DOI: 10.1158/1078-0432.CCR-21-2404]

11   **Kasi PM**, Dayyani F, Morris VK, Kopetz S, Aleshin A. Tumor-informed assessment of molecular residual disease and its incorporation into practice for patients with early and advanced-stage colorectal cancer (CRC-MRD Consortia). *J Clin Oncol* 2020; **38(15_suppl)**: 4108-4108 [DOI: 10.1200/jco.2020.38.15_suppl.4108]

12   **Cremolini C**, Rossini D, Dell'Aquila E, Lonardi S, Conca E, Del Re M, Busico A, Pietrantonio F, Danesi R, Aprile G, Tamburini E, Barone C, Masi G, Pantano F, Pucci F, Corsi DC, Pella N, Bergamo F, Rofi E, Barbara C, Falcone A, Santini D. Rechallenge for Patients With RAS and BRAF Wild-Type Metastatic Colorectal Cancer With Acquired Resistance to First-line Cetuximab and Irinotecan: A Phase 2 Single-Arm Clinical Trial. *JAMA Oncol* 2019; **5**: 343-350 [PMID: 30476968 DOI: 10.1001/jamaoncol.2018.5080]

13   **Bando H**, Kagawa Y, Kato T, Akagi K, Denda T, Nishina T, Komatsu Y, Oki E, Kudo T, Kumamoto H, Yamanaka T, Yoshino T. A multicentre, prospective study of plasma circulating tumour DNA test for detecting RAS mutation in patients with metastatic colorectal cancer. *Br J Cancer* 2019; **120**: 982-986 [PMID: 31015557 DOI: 10.1038/s41416-019-0457-y]

14   **García-Foncillas J**, Alba E, Aranda E, Díaz-Rubio E, López-López R, Tabernero J, Vivancos A. Incorporating BEAMing technology as a liquid biopsy into clinical practice for the management of colorectal cancer patients: an expert taskforce review. *Ann Oncol* 2017; **28**: 2943-2949 [PMID: 28945877 DOI: 10.1093/annonc/mdx501]

15   **Silva J**, Garcia V, Rodriguez M, Compte M, Cisneros E, Veguillas P, Garcia JM, Dominguez G, Campos-Martin Y, Cuevas J, Peña C, Herrera M, Diaz R, Mohammed N, Bonilla F. Analysis of exosome release and its prognostic value in human colorectal cancer. *Genes Chromosomes Cancer* 2012; **51**: 409-418 [PMID: 22420032 DOI: 10.1002/gcc.21926]

16   **Matsumura T**, Sugimachi K, Iinuma H, Takahashi Y, Kurashige J, Sawada G, Ueda M, Uchi R, Ueo H, Takano Y, Shinden Y, Eguchi H, Yamamoto H, Doki Y, Mori M, Ochiya T, Mimori K. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br J Cancer* 2015; **113**: 275-281 [PMID: 26057451 DOI: 10.1038/bjc.2015.201]

17   **Dong L**, Lin W, Qi P, Xu MD, Wu X, Ni S, Huang D, Weng WW, Tan C, Sheng W, Zhou X, Du X. Circulating Long RNAs in Serum Extracellular Vesicles: Their Characterization and Potential Application as Biomarkers for Diagnosis of Colorectal Cancer. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 1158-1166 [PMID: 27197301 DOI: 10.1158/1055-9965.EPI-16-0006]

18   **Galamb O**, Barták BK, Kalmár A, Nagy ZB, Szigeti KA, Tulassay Z, Igaz P, Molnár B. Diagnostic and prognostic potential of tissue and circulating long non-coding RNAs in colorectal tumors. *World J Gastroenterol* 2019; **25**: 5026-5048 [PMID: 31558855 DOI: 10.3748/wjg.v25.i34.5026]

19   **Zhou B**, Xu K, Zheng X, Chen T, Wang J, Song Y, Shao Y, Zheng S. Application of exosomes as liquid biopsy in clinical diagnosis. *Signal Transduct Target Ther* 2020; **5**: 144 [PMID: 32747657 DOI: 10.1038/s41392-020-00258-9]

20   **Cai Z**, Wang Z, Liu C, Shi D, Li D, Zheng M, Han-Zhang H, Lizaso A, Xiang J, Lv J, Wu W, Zhang Z, Yuan F, He S, Sun J. Detection of Microsatellite Instability from Circulating Tumor DNA by Targeted Deep Sequencing. *J Mol Diagn* 2020; **22**: 860-870 [PMID: 32428677 DOI: 10.1016/j.jmoldx.2020.04.210]

21   **Gandara DR**, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, Rittmeyer A, Fehrenbacher L, Otto G, Malboeuf C, Lieber DS, Lipson D, Silterra J, Amler L, Riehl T, Cummings CA, Hegde PS, Sandler A, Ballinger M, Fabrizio D, Mok T, Shames DS. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med* 2018; **24**: 1441-1448 [PMID: 30082870 DOI: 10.1038/s41591-018-0134-3]

22   **Salvianti F**, Gelmini S, Mancini I, Pazzagli M, Pillozzi S, Giommoni E, Brugia M, Di Costanzo F, Galardi F, De Luca F, Castiglione F, Messerini L, Pinzani P, Antonuzzo L. Circulating tumour cells and cell-free DNA as a prognostic factor in metastatic colorectal cancer: the OMITERC prospective study. *Br J Cancer* 2021; **125**: 94-100 [PMID: 33953347 DOI: 10.1038/s41416-021-01399-6]

Novel approaches in search for biomarkers of cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) arises from the ductular epithelium of the biliary tree, either within the liver (intrahepatic CCA) or more commonly from the extrahepatic bile ducts (extrahepatic CCA). This disease has a poor prognosis and a growing worldwide prevalence. The poor outcomes of CCA are partially explained by the fact that a final diagnosis is challenging, especially the differential diagnosis between hepatocellular carcinoma and intrahepatic CCA, or distal CCA and pancreatic head adenocarcinoma. Most patients present with an advanced disease, unresectable disease, and there is a lack in non-surgical therapeutic modalities. Not least, there is an acute lack of prognostic biomarkers which further complicates disease management. Therefore, there is a dire need to find alternative diagnostic and follow-up pathways that can lead to an accurate

result, either singlehandedly or combined with other methods. In the "-omics" era, this goal can be attained by various means, as it has been successfully demonstrated in other primary tumors. Numerous variants can reach a biomarker status ranging from circulating nucleic acids to proteins, metabolites, extracellular vesicles, and ultimately circulating tumor cells. However, given the relatively heterogeneous data, extracting clinical meaning from the inconsequential noise might become a tall task. The current review aims to navigate the nascent waters of the non-invasive approach to CCA and provide an evidence-based input to aid clinical decisions and provide grounds for future research.

Core Tip: The current review paper aims to critically analyze the most recent developments in non-invasive cholangiocarcinoma diagnosis and prognosis. The article takes an in-depth look at the fields of circulating nucleic acids, proteomic and metabolomic-derived biomarkers, extracellular vesicles, and circulating tumor cells in an attempt to outline promising results for future research and clinical use.

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from the biliary epithelial cells. The latest World Health Organization Classification of Tumors-Digestive System Tumors acknowledges the heterogeneous nature of CCA, emphasizing the importance of tumor localization. In this matter, there are two main types of CCAs: Intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA) [including both: Perihilar CCA (pCCA) and distal (dCCA)], featuring different aspects in etiology, molecular alterations, pathogenesis, behavior, potential diagnostic or prognostic biomarkers and hence a different clinical management[1].

iCCAs represent approximately 10%-15% of liver tumors and the second primary liver malignancy, after hepatocellular carcinoma (HCC)[2], while eCCAs account for 0.5-2 cases/10.000 person-years[3]. Although considered a relatively rare type of cancer, the incidence of CCA is rising in most geographic areas[4]. Both HCC and iCCA, although they are considered different diseases, do share some common risk factors including hepatitis B or C, non-biliary hepatic cirrhosis, alcoholic and non-alcoholic steato-hepatitis, or metabolic syndrome. On the other hand, eCCA typically occurs in conditions associated with chronic biliary inflammation, such as primary sclerosing cholangitis, lithiasis, cysts, or liver fluke infections. In most cases, the exact etiology remains difficult to pinpoint[5].

To this point, CCA is notoriously difficult to diagnose. Diagnosing these tumors requires the correlation of clinical, imaging, and, when available, histopathologic data. In terms of treatment, surgical resection with curative intent remains the best option. However, most patients with CCA (approximately 70%) are diagnosed at late stages due to lack of specific symptoms[6]. Mortality rates are high, and thus the prognosis is poor[7], especially in the case of large tumors, satellite nodules, vascular or lymphatic invasion, positive resection margins, or advanced pathological tumor-node-metastasis stages (TNM)[8,9]. For surgically resectable tumors, the 5-year survival rate reaches 20%-30%, but the percentage drops to a bitter 0% for the rest of the cases[10]. After surgery, the recurrence rate is relatively high, reaching from 49% to 70%[11] and relapse occurs early, typically within 2 or 3 years after surgery[8].

These circumstances emphasize the necessity of novel, clinical-suited tools that would serve for early diagnosis, as prognostic indicators or in treatment guidance, such as biomarkers.

Biomarkers were defined by the Food and Drug Administration-National Institute of Health Biomarker Working Group back in 2016 as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers.". While α-fetoprotein (AFP) is the most convenient and non-invasive serum

biomarker for detecting HCC, elevated AFP was observed only in approximately 20% of a subgroup of CCA, namely iCCA patients[12].

The charbohydrate antigen 19-9 (CA19-9) is currently used worldwide in clinical practice as a non-specific serum marker for orientation in diagnosing CCA, but it bears a low sensitivity in European patients[13]. In terms of prognosis there are some validated tools that are useful in the clinical practice. These markers are not specific for CCA but rather apply to all human malignancies. Tumor size and differentiation, vascular involvement, lymph node status, margin status and presence of occult metastasis were all shown to be good predictors for overall survival (OS) for both iCCA and eCCA[14].

One option could be the study of tumor tissue in search of novel biomarkers. This strategy appeared to be fruitful, as several tumor tissue-based biomarkers were already identified. Mutations in TP53 and KRAS proto-oncogene are associated with an impaired outcome-lower OS and higher tumor recurrence than other mutations in resected CCA while several other genetic signatures with prognostic potential include epidermal growth factor receptor (EGFR), mucin 1 (MUC1), MUC4, and fascin (FSCN) expression[15]. Moreover, alteration in targetable pathways [*e.g.*, fibroblast growth factor receptor 2 gene (FGFR2) involved in MAP kinase signaling, isocitrate dehydrogenase 1 and 2 (IDH1, IDH2)] were also depicted in CCA patients[16] and currently, several clinical trials are actively recruiting patients. Nevertheless, several microRNAs (miRs) expressions in tissue or deregulated immune responses [expression levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4), forkhead box P3 (FOXP3), and programmed death-ligand 1 (PD-L1)] might have predictive capabilities in CCA patients[17,18]. Many other diagnostic and prognostic tissue-derived biomarkers have already been previously described[19].

Unfortunately, biopsy collection for tissue analysis is not an ideal biospecimen for biomarker assessment and translation to clinical practice. Although it offers absolute insights into tumor biology, the collection procedure presents several caveats and poses the risk of serious clinical complications [20]. As an alternative to tissue biopsy, a much more reliable biospecimen, already implemented in the clinical practice with several advantages over tissue, is the liquid biopsy (blood). Serum, plasma, or urine, collected non-invasively using well-established low-cost techniques are considered "ideal fluids" in biomarker research. Moreover, liquid biopsy encloses molecules from the whole body, and a single sample can offer a wide range of information and is enough for multiple measurements.

The current review aims to explore the nascent waters of the non-invasive biomarkers reported for CCA by taking an in-depth look at the fields of circulating nucleic acids, proteomic and metabolomic-derived biomarkers, extracellular vesicles, and circulating tumor cells (Figure 1) and provide an evidence-based input which could provide grounds for future research to pave the way for prospective validation and translation into the clinical practice of novel biomarkers. In this review the term CCA will make reference to all types of CCA, while the terms iCCA, hCCA, pCCA, and dCCA will stand for intrahepatic, hilar, perihilar and distal CCA, respectively.

CIRCULATING NUCLEIC ACIDS

Circulating nucleic acids represent snippets of genetic material, either DNA (cell-free DNA–cfDNA) or RNA (usually miR), reaching various fluid compartments (serum, urine, bile) through active cellular export or following cell death. The road from bench to bedside for circulating nucleic acids has taken a relatively long time and has not quite reached the point of clinical applicability in cancer diagnosis. However, more than four decades have passed between the initial proof-of-concept[21] and present-day genome-wide cfDNA mutational integration[22]. The world of cfDNA and miRs seems to be emerging more promising than ever, as the highly effervescent field has started to deliver on the early expectations. Moreover, CCA might provide a unique setting for the method to flourish: A conventional diagnostic challenge, sometimes a hard to biopsy tumor, all while having a relatively underdeveloped therapeutic arsenal.

CfDNA–the mutational fingerprint

The analysis of circulating cfDNA can provide a quick, complete, and non-invasive mutational profile of any tumor, by amplifying each mutation encountered throughout the tumor burden. The method reflects the entirety of mutations, thus not being the subject of selection bias in the case of heterogeneous cancers. More specifically, tissue samples can provide the mutational palette only for the available specimen. Therefore, the genetic fingerprint of a tumor might be incomplete, as metastases or distant regions of a tumor might have additional alterations. Consequently, cfDNA provides (at least in principle) a better understanding of the disease, with a substantial impact in disease management, from diagnostics to guide therapeutic choices.

This concept has been recently validated for CCA, using plasma samples of patients with fully characterized mutation status[23]. According to the study design, 31 mutations in the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes were screened using multiplex polymerase chain reaction (PCR) and further quantified. The results were then compared with the mutational profile of the primary tumor, resulting in a perfect match. These results were partially reinforced by the work of a German team that performed the deep sequencing of 15 genes involved in CCA (*n* = 32), revealing a 74% overall blood-tissue sample
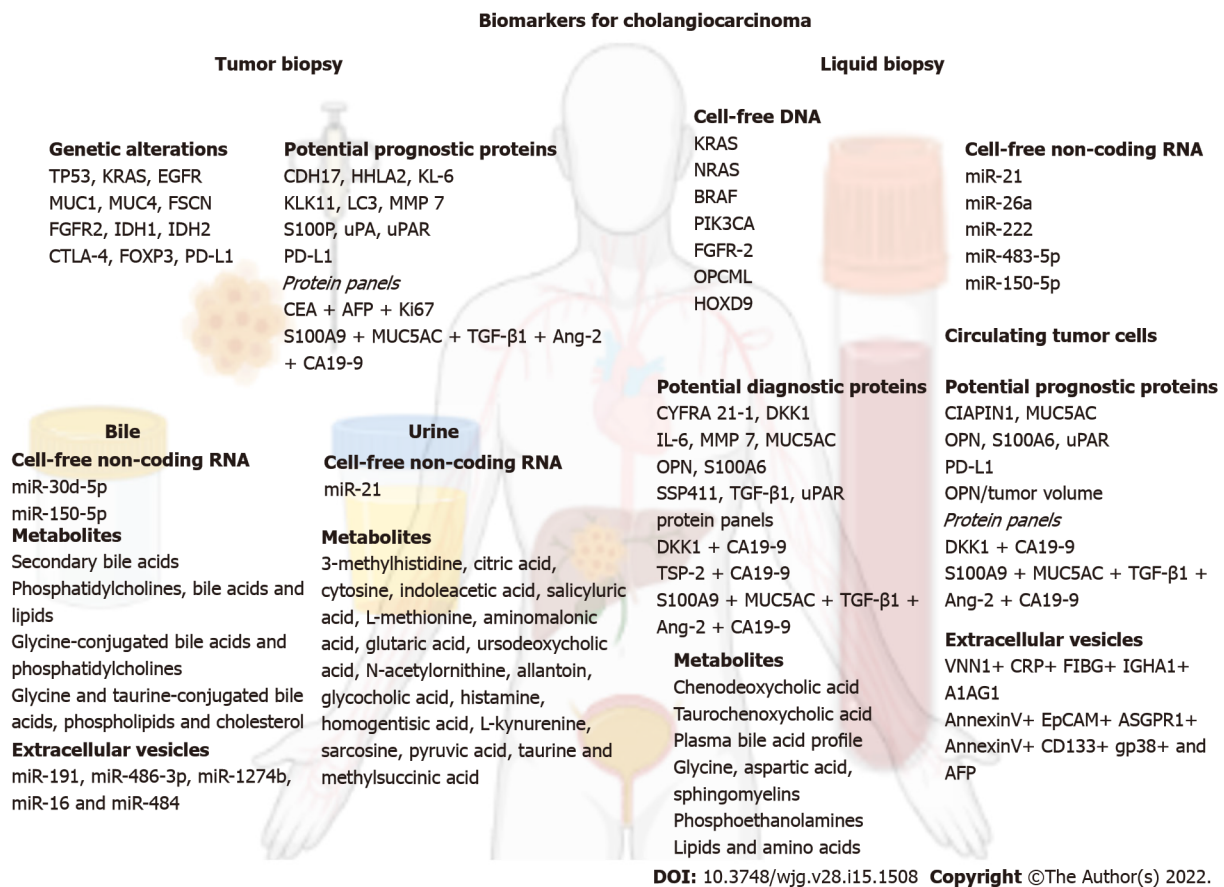
Figure 1 An overview on the biomarkers for cholangiocarcinoma. Created with biorender.com. A1AG1: Alpha-1 acid glycoprotein; AFP: Alpha fetoprotein; Ang-2: Angiopoietin-2; ASGPR1: Asialoglycoprotein receptor 1; CA19-9: Charbohydrate antigen 19-9; CDH17: Cadherin-17; CEA: Carcinoembryonic antigen; CIAPIN1: Cytokine-induced apoptosis inhibitor 1; CRP: C-reactive protein; CTLA-4: Cytotoxic T-lymphocyte antigen 4; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf-1; EGFR: Epidermal growth factor receptor; EpCAM: Epithelial cell adhesion molecule; FGFR2: Fibroblast growth factor receptor 2; FIBG: Fibrinogen gamma chain; FOXP3: Forkhead box P3; FSCN: Fascin; HHLA2: Human endogenous retrovirus-H long terminal repeat-associating protein 2; IDH1: Isocitrate dehydrogenase 1; IDH2: Isocitrate dehydrogenase 2; IGHA1: Immunoglobulin heavy constant alpha 1; IL-6: Interleukin 6; Ki67: Proliferation marker protein Ki67; KL-6: Krebs von den Lungen 6; KLK11: Kallikrein related peptidase 11; LC3: Microtubule-associated protein 1A/1B-light chain 3; MMP-7: Metalloproteinase 7; MUC1: Mucin 1; MUC4: Mucin 4; MUC5AC: Mucin 5AC; OPN: Osteopontin; PD-L1: Programmed death-ligand 1; S100A6: S100 calcium-binding protein A6; S100A9: S100 calcium-binding protein A9; S100P: Tissue protein S100P; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPA: Urokinase-type plasminogen activator; uPAR: Urokinase-type plasminogen activator receptor; VNN1: Pantetheinase.

concordance and 92% for intrahepatic tumors. Moreover, the patients were followed throughout chemotherapy, during which 63% of the patients had their mutational fingerprint altered[24]. This finding might have particular implications regarding treatment selection, especially in the case of loss of response. There is evidence of resistance to BGJ398, a pan-FGFR inhibitor, due to *de novo* point mutations in the FGFR-2 kinase domain, revealed by cfDNA analysis[25].

Another promising subfield of cfDNA in CCA is the study of cell-free epigenetics. A recently published report, which analyzed 40 samples of each patient group, hints towards distinct methylation profiles between benign biliary tract disease (BTD) and CCA. The methylation pattern of opioid-binding protein/cell adhesion molecule (OPCML) and homeobox D9 (HOXD9) had a promising discriminative potential, with an area under the receiver operating characteristic (AUROC) of 0.85 for diagnosing CCA [26].

However, we believe that, to this point, there is a dire need for more data to support these initial findings. Barriers regarding study design: Method synchronization, number of patients included, data heterogeneity, cost, and lack of validation prevent their use in clinical settings, while also preventing the funding of large-scale translational endeavors.

Cell-free non-coding RNA

Research in the past decade has revealed an increasing role of miRs as cancer biomarkers for multiple primary tumors, including CCA[27-31]. There are several qualities that, at least in theory, favor miRs as useful biomarkers: Relative specificity, long-term stability, presence in multiple fluids, as well as relative ease of detection and amplification through ever more accessible PCR techniques[19]. To this point, numerous studies[32-36] have investigated the role of miRs in CCA, some showing substantial promise [37-41]. These studies are briefly analyzed in Table 1. The viability of miRs as biomarkers in CCA was

Table 1 The role of microRNAs as diagnostic and prognostic biomarkers in cholangiocarcinoma

Circulating microRNA	Biofluid	Comparison	Statistics	Discriminant specificity	Valuable considerations	Subjective rating
miR-21	Increased in serum[27,28]; plasma[34]; urine[35]	iCC (*n* = 74), HC (*n* = 74)[27]; CCA (*n* = 11), HC (*n* = 9)[28]. iCC (*n* = 25), HC (*n* = 7); CCA (*n* = 22), HC (*n* = 21)	AUROC *vs* HC: Serum: 0.91[27]; serum: 0.80[28]; plasma 0.94[34]. Combined miR-21 + miR 192. Urine: 0.85 [35]	LOW. Also increased in HCC[36] and other malignancies[37,38]	Corelates well with tumor stage and survival[39]. Most data support	Useful
miR-150-5p	Decreased in serum and bile[40]	CCA (*n* = 28), PSC (*n* = 30), HC (*n* = 50)	Significantly decreased *vs* HC and PSC[40] association with	LOW. Upregulation suppresses tumor progression in colorectal cancer[41]	Appears to correlate with tumor staging. Added value of the CA19-9 combination. Contradictory findings: Report of being upregulated in iCCA (AUROC: 0.76)[27]	Debatable
miR-26a	Increased in serum[29]	CCA (*n* = 66), HC (*n* = 66)	AUROC *vs* HC: 0.90 [29]	Moderate involved in HCC[30]	Correlates well with tumor stage, metastases, differentiation, and survival. Reliable decrease following curative surgery[29]	Promising
miR-30d-5p	Increased in bile[31]	CCA (*n* = 48), benign BTD (*n* = 58)	AUROC *vs* benign biliary obstruction 0.730[31]	Moderate downregulated in gastric cancer[32]	Increased sensitivity and specificity compared to CA19-9	Debatable
miR-222; miR-483-5p	Increased in serum[33]	CCA (*n* = 70), PSC (*n* = 70), HC (*n* = 70)	AUROC *vs* PSC; miR-222: 0.71; miR-483-5p: 0.70 combined miR-222 and 483-5p: 0.74 [33]	No evidence of overlap with other cancers	Might be useful for monitoring patients with PSC	Promising

AUROC: Area under a receiver operating characteristic; CA19-9: Carcinoembrionic antigen 19-9; CCA: Cholangiocarcinoma; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; miRs: Micro RNAs; PSC: Primary sclerosing cholangitis.

tested in two meta-analyses, each including approximately 500 patients and testing the diagnostic capabilities of the cell-free non-coding RNA method, without focusing on specific miRs. Overall, the results were promising, with an AUROC ranging between 0.88 and 0.90 for CCA detection[42,43].

However, there are some nuances in the study of miRs, which are worth addressing since the clinical future of the method might be at stake. Of critical relevance is the problem of specificity. Most biomarkers fare relatively well against healthy controls (HC), which is helpful for initial validation, yet far from desirable in a clinical scenario where the setting is less straightforward. This issue has been partially addressed in some study designs by comparisons with other benign BTD conditions, probably the most relevant being primary sclerosing cholangitis (PSC), which shares a common natural history pathway with CCA. However, in such conditions, the specificity and AUROCs tend to drop at least by 0.10-0.20 (as shown in Table 1). Consequently, their diagnostic biomarker value remains only slightly above the threshold for utility in the clinical scenario where the discriminative capabilities were most sought after. Moreover, there is the issue of overlapping with other cancers, which might further complicate the matter. In response, some designs have tried to implement a panel of up to eight miRs to generate distinct profiles depending on CCA subtypes (*n* = 14) and tumor progression[44].

The use of circulating nucleic acids in CCA diagnosis and prognosis is undoubtedly promising. Nevertheless, the field is still nascent, and most of the data come from studies with heterogeneous designs, most of which are proof-of-concept. Therefore, a potential research direction might be to stimulate reproducibility instead of novelty to provide the grounds for a quicker clinical application.

PROTEINS

Protein-based biomarkers in the clinical practice

Proteomics is a rapidly growing field of biomedical research in the postgenomic era, given the ever-expanding role of personalized medicine. Proteome-based biomarker studies target proteins that could serve as agents to fit a patient's molecular profile in the clinical practice for diagnostic, prognostic, and predictive molecules, their levels being measured from serum samples usually by ELISA.

There are three protein-based biomarkers currently used in the clinical practice towards assisting CCA diagnosis and prognosis: CA19-9 and CA125, and carcinoembryonic antigen (CEA)[45,46].

CA19-9 is a circulating high molecular weight glycoprotein produced by the biliary duct and pancreatic cells and secreted by the gastric and colonic epithelia. Up to 7% of the general population is not producing CA19-9 because of blood cell Lewis antigen deficiency. For CCA, CA19-9 is by far the most frequently used biomarker. Concerning CCA diagnosis, CA19-9 showed a somewhat limited diagnostic accuracy, with following performances: Sensitivity: 72% and specificity: 84%[13]. Hence its promise resides in assessing CCA prognosis. As recently reviewed by Lang *et al*[45] CA19-9 appears to be an independent prognostic biomarker associated with treatment outcome, as elevated CA19-9 serum levels pre- and postoperatively after systemic therapy show impaired OS. Nevertheless, several factors hamper CA19-9 use as a unique CCA prognostic biomarker[47], thus making its clinical use tumor-associated rather than tumor-specific.

Also known as MUC16, CA125 is the largest membrane-associated mucin, which is also, a glycoprotein. Being a well-known biomarker, CA125 is primary used for the ovarian cancer clinical management[48]. CA125 showed incipient potential diagnostic and prognostic value towards clinical management of CCA[45]. However, CA125 proves its predictive power only in combination with other biomarkers, such as CA19-9, CAE and AFP.

Being produced by the gastrointestinal tissue during fetal development, CEA is a cell surface glycoprotein and functions as an intracellular adhesion molecule. In clinical practice, CEA is extensively used in colorectal cancer monitoring[49]. CEA proved its potential value as a diagnostic biomarker, with rages of sensitivity reported between 40% and 79%, and specificity between 48% and 90%. CEA was also reported as a prognostic indicator for CCA, with expanded predictive capabilities in several biomarker combinations, such as with CA19-9[45].

The three glycoproteins are the most used biomarkers in the clinical management of CCA, and their role is to assist rather than provide a definite diagnostic or prognostic statement. Various other protein-based biomarker candidates reported as single molecules, combined with CA19-9 or as biomarker panels, have been spotlighted in several CCA studies. Towards identifying the potential biomarkers, several approaches have been used. With respect to the study design, CCA patient samples have been compared to (1) Only HC; (2) Only to benign BTD; (3) To benign BTD and HC; and (4) To other disease related conditions and HC. Other studies were interested only in searching biomarkers for iCCA and only one approach was headed towards subtypes of CCA, such as perihilar iCCA, hCCA, and eCCA. While the biospecimens are limited to serum and tissue, and the methods to ELISA or immunohisto-chemistry, the number of samples included appears to be very heterogenous, ranging from around 20 to up to over 200. The proteins associated with favorable diagnostic and with poor prognostic, potential protein-based biomarkers, are deciphered in Tables 2 and 3.

Proteins associated with favorable diagnostic potential in CCA patients

Multiple proteins appeared to have a role in CCA diagnosis, typically showing increased serum levels. These findings were reported in studies using serum as biospecimen and ELISA assays for their absolute quantification (Table 2). Such examples are osteopontin (OPN)[50] and S100 calcium-binding protein A6 (S100A6)[51] which efficiently discriminated between CCA and HC, and dickkopf-1 (DKK1) between iCCA and HC[52]. Studies reporting serum cytokine interleukin 6 (IL-6)[53], spermatogenesis-associated protein 20 (SSP411)[54] and MUC5AC[55] also included groups of metastatic liver cancer, HCC, and benign BTD disease. Metalloproteinase 7 (MMP-7) was assessed only in groups of CCA *vs* benign BTD[56,57].

After several reports, cytokeratin 19 fragment (CYFRA 21-1) was included in a comprehensive meta-analysis[58] and the pooled diagnostic indices showed a sensitivity of 81% and a specificity of 86% for iCCA diagnosis. More recently, the urokinase-type plasminogen activator receptor (uPAR)[59], reported as a single protein-based biomarker, proved to be a reliable tool for differentiating CCA from HC with sensitivity of 95% and specificity close to 90%, while transforming growth factor-β1 (TGF-β1) appears to help distinguishing CCA from other pro-inflammatory conditions and HC.

Moreover, the combination of MMP-7[57], DKK1[60], thrombospondin-2 (TSP-2)[61] and uPAR[59] assessed together with CA19-9 showed higher values of sensitivity and specificity than the markers measured individually to diagnose CCA patients. Not least, a biomarker panel consisting of five proteins investigated in a decision tree algorithms based study, namely S100A9, MUC5AC, TGF-β1, angiopoietin-2 (Ang-2), and CA19-9, showed to have the greatest diagnostic potential among all mentioned proteins towards CCA *vs* HC (sensitivity: 95%, specificity: 90%) and towards CCA *vs* non-CCA (sensitivity: 70%, specificity: 83%) differentiation[62].

Proteins associated with poor outcome in CCA patients

Concerning prognosis, several protein-based potential biomarkers have shown increased levels in CCA, most frequently by employing immunohistochemistry in tissue samples. The serum has also emerged as a biospecimen towards prognostic biomarkers exploration (Table 3). As such, high levels of tissue Krebs von den Lungen 6 (KL-6 mucin)[63], cadherin-17 (CDH17)[64], kallikrein-11[65], uPAR[59] and high levels of serum cytokine-induced apoptosis inhibitor 1 (CIAPIN1)[66], MUC5AC[55], OPN[50], S100A6 [67] and uPAR[59,68] were found adverse prognostic factors for CCA patient's survival. Subjected to meta-analysis, high levels of tissue PD-L1[17] and tissue protein S100P[69] were also proposed as potential prognostic markers of CCA. Out of a protein multimarker panel consisting of serum S100A9,

Table 2 Proteins associated with favorable cholangiocarcinoma diagnostic potential

Protein	Comparison	SEN (%)	SPE (%)	AUC	Ref.
	Tissue				
CYFRA 21-1	iCCA (n = 217) *vs* HC (n = 514) meta-analysis	81.0	86.0	0.904	[58]
DKK1	iCCA (n = 37) *vs* HC (n = 50)	75.7	100.0	0.872	[52]
DKK1 + CA19-9	iCCA(n = 79) *vs* HC (n = 160)	74.7	56.3	0.793	[60]
IL-6	CCA (n = 26), HCC (n = 26) and HC (n = 23)	73.0	92.0	0.875	[53]
MMP-7	CCA (n = 44) *vs* benign BTD (n = 36)	76.3	46.8	0.730	[56]
	CCA (n = 59) *vs* benign BTD (n = 128)	75.0	78.0	0.840	[57]
MUC5AC	CCA (n = 49), benign BTD (n = 23), HC (n = 16)	71.0	94.7	0.909	[55]
OPN	CCA (n = 107) *vs* HC (n = 55)	87.5	100.0	0.964	[50]
S100A6	CCA (n = 112) *vs* HC (n = 42)	86.2	90.9	0.909	[51]
SSP411	CCA (n = 30), benign BTD (n = 13) and HC (n = 23)	90.0	83.3	0.913	[54]
TGF-β1	CCA (n = 45), other disease conditions related inflammation (n = 25) and HC (n = 45)	71.1	68.9	0.668	[78]
TSP-2 + CA19-9	dCCA (n = 51), pancreatic ductal adenocarcinoma (n = 52), benign pancreatic diseases (n = 27) and HC (n = 52)	79.0	96.0	0.920	[61]
uPAR	CCA (n = 118), and HC (n = 76)	95.3	89.7	0.969	[59]
Biomarker panel: S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9	CCA (n = 40), non-CCA (n = 40) and HC (n = 40)	95.0	90.0	0.975	[62]

AUC: Area under the curve; BTD: Biliary tract disease; CCA: Cholangiocarcinoma; dCCA: Distal CCA; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; SEN: Sensitivity; SPE: Specificity; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf 1; IL-6: Interleukin 6; MMP-7: Metalloproteinase 7; MUC5AC: Mucin 5AC; OPN: Osteopontin; S100A6: S100 calcium binding protein A6; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPAR: Urokinase-type plasminogen activator receptor; S100A9: S100 calcium binding protein A9; Ang-2: Angiopoietin-2.

MUC5AC, TGF-β1, Ang-2, and CA19-9, serum levels of TGF-β1 and Ang-2 provided predictive potential for both metastasis and TNM stage prognosis in CCA patients[62].

For patients with tumors of combined HCC and CCA (cHCC-CC), microtubule-associated protein 1A/1B-light chain 3 (LC3) increased tissue expression was found to predict postresection OS (5-year OS, 61.2%) and disease-free survival (74.6%)[70].

iCCA prognostic biomarkers were also of particular interest in some studies. High tissue levels of urokinase-type plasminogen activator (uPa)[71], MMP-7[72,73] and human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2), reported from a recent meta-analysis[74], were associated with adverse outcomes in iCCA patients. High serum DKK1 in combination with CA19-9 was independently associated with shorter survival[60]. Recently, Qiang *et al*[75] found that the biomarker panel consisting of CEA, AFP, and proliferation marker protein Ki67 are significant prognostic indicators in iCC patients.

Proteins that showed decreased levels in association with CCA were PD-L1 and OPN. The lack of serum PD-L1 level normalization after surgery seems to identify patients at high risk for recurrence and adverse outcomes[76]. By applying an innovative approach, decreased serum OPN *per* tumor volume was associated with invasive behavior and early recurrence of iCCA[77].

There is vast evidence of protein-based biomarkers reported in CCA diagnosis and prognosis, but only CA19-9 and CEA are currently employed in routine clinical practice. The data above reveals exciting results for new potential protein-based biomarkers used as single molecules (*e.g.*, uPAR) or biomarker panels (*e.g.*, S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9) for CCA.

Extrapolating from proteomic-derived biomarker studies in other diseases, it appears that using multiple-molecule panels instead of individual proteins provides better predictive results and shows more promise for a translation to clinical practice. However, future validation studies on large patient cohorts are needed towards establishing the real applicability and the subsequent translation of these

Table 3 Proteins associated with poor outcome in cholangiocarcinoma patients

Protein	Comparison	Outcome	Ref.
Tissue			
CDH17	CCA (*n* = 180)	High CDH17 was associated with a worse OS and recurrence-free survival	[64]
HHLA2	iCCA (*n* = 218) meta-analysis	High HHLA2 expression was significantly associated with shorter OS	[74]
KL-6	CCA (*n* = 21), cHCC-CCA (*n* = 12), HCC (*n* = 78)	A key molecule for tumor cell adhesion and invasion	[63]
KLK11	CCA and adjacent normal tissues (*n* = 18)	OS of CCA patients with a high expression of KLK11 was significantly shorter than those with a low expression of KLK11 (414 d *vs* 809 d, respectively; *P* = 0.048)	[65]
LC3	cHCC-CC (*n* = 40)	The 5-yr OS and disease-free survival rates were 61.2% and 74.6% in high LC3 expression patients and 0% and 0% in those with low LC3 expression	[70]
MMP-7	Perihilar iCCA, hCCA, and eCCA (*n* = 66)	Patients with moderate to marked expression of MMP-7 had a significantly poorer prognosis, as compared to those with negative to focal expression	[72]
	iCCA (*n* = 35)	The 5-yr survival rates of MMP-7(+) and MMP-7(−) patients were 72.7% and 18.3%, respectively	[73]
PD-L1	CCA (*n* = 2012) meta-analysis	Overexpression of PD-L1 was significantly associated with worse OS	[17]
S100P	CCA (*n* = 1925) meta-analysis	S100 calcium binding protein P overexpression was associated with poor OS	[69]
uPa	iCCA (*n* = 174)	High uPa expression was correlated with lymphatic invasion and metastasis of CCA patients	[71]
uPAR	CCA (*n* = 108) *vs* normal tissue (*n* = 108)	The median OS was 890 d for patients with uPAR positive vs 1.321 d for patients with uPAR negative	[59]
Biomarker panel: CEA, AFP, and Ki67	iCCA (*n* = 92)	higher AFP, CEA, and Ki67, as well as more advanced TNM staging were associated with worse OS	[75]
Serum			
CIAPIN1	CCA (*n* = 159) *vs* HC (*n* = 93)	Higher CIAPIN1 level was significantly associated with shorter OS time	[66]
DKK1 + CA19-9	iCCA (*n* = 79) *vs* HC (*n* = 160)	DKK-1 in combination with CA19-9 showed a better diagnostic performance than CA19-9 alone; low DKK-1 and CA19-9 were associated with longer OS	[60]
MUC5AC	CCA (*n* = 49), benign BTD (*n* = 23), HC (*n* = 16)	High MUC5AC level was related to a worse prognosis compared with patients with lower levels, with 3-yr survival rates of 21.5% and 59.3%, respectively	[55]
OPN	CCA (*n* = 107) *vs* HC (*n* = 55)	Poor postoperative survival	[50]
OPN/tumor volume	iCCA (*n* = 124)	Low circulating OPN *per* tumor volume was associated with shorter OS and disease-free survival	[77]
PD-L1	CCA (*n* = 73) *vs* HC (*n* = 42)	Low PD-L1 levels displayed a strong trend towards an impaired prognosis	[76]
S100A6	CCA (*n* = 112) *vs* HC (*n* = 42)	S100A6 potential was like those of the clinically established biomarkers CEA and CA19-9	[67]
uPAR	CCA (*n* = 168)	Baseline level of uPAR was an independent predictor of survival; a high level of uPAR after 2 cycles of chemotherapy was associated with poor survival	[68]
	CCA (*n* = 117), HC (*n* = 76)	Multivariate Cox-regression analysis revealed	[59]

		circulating uPAR levels as an independent prognostic marker following biliary tract cancer resection	
Biomarker panel: S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9	CCA (*n* = 40), and non-CCA patients (*n* = 40) and HC (*n* = 40)	TGF-β1 and Ang-2 are predictors of higher TNM stages	[62]

BTD: Biliary tract disease; CCA: Cholangiocarcinoma; cHCC-CC: Combined hepatocellular carcinoma and CCA; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; OS: Overall survival; CDH17: Cadherin-17; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf 1; IL-6: Interleukin 6; MMP-7: Metalloproteinase 7; MUC5AC: Mucin 5AC; OPN: Osteopontin; S100A6: S100 calcium binding protein A6; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPAR: Urokinase-type plasminogen activator receptor; S100A9: S100 calcium binding protein A9; Ang-2: Angiopoietin-2; PD-L1: Programmed death-ligand 1; CIAPIN1: Serum cytokine-induced apoptosis inhibitor 1; AFP: Alpha-feto protein; LC3: Microtubule-associated protein 1A/1B-light chain 3; KLK11: Kallikrein related peptidase 11; KL-6: Mucin KL-6; HHLA2: Human endogenous retrovirus-H long terminal repeat-associating protein 2.

biomarkers into the clinical practice.

METABOLITES

Metabolomics, another branch of omics-derived technologies, analyzes low molecular weight metabolites (< 1500 Da) in various biological fluids. One of the hallmarks of cancer is energy metabolism reprogramming. In order to promote cancer survival and subsequently cancer growth, there are several shifts in normal metabolic pathways (*e.g.*, a higher glucose uptake rate and an increase in lactate production)[78,79]. A different or "wiser" use of metabolic pathways in cancer cells leads to the release of several metabolites in various body fluids, providing an opportunity for diagnosis and monitoring. Metabolic profiling is, therefore, a promising approach for the identification of potential biomarkers in several cancers, including CCA[80]. To date, several studies have investigated the potential of metabolomics in CCA diagnosis or prognosis in various body fluids.

We believe that investigating the molecular composition of the bile could provide more crucial information than other fluids due to at least two reasons. Firstly, it could unravel mechanistic information regarding the pathological alteration of the biliary epithelium. Secondly, it could identify biomarkers from nearby tumor cells, markers that might or might not be present in other body fluids. Several metabolite profiling studies of human bile have been performed over the past few years. One such study reported a reduction in the proportion of secondary bile acids in patients with CCA compared to those with biliary tract stones and healthy individuals[81]. Another study showed that changes in phosphatidylcholines, bile acids, and lipids could discriminate CCA from PSC and benign BTD (sensitivity: 88.9%; specificity: 78.1%)[82]. When comparing inoperable eCCA to non-malignant, non-cholestatic biliary diseases (including PSC), CCA was associated with increased levels of glycine-conjugated bile acids and phosphatidylcholines. Moreover, constructed models could discriminate CCA patients from those with non-malignant biliary diseases with an 80% sensitivity and 95% specificity: 95%[83].

Unfortunately, the impact of cholestasis on the metabolic profile was not investigated, and it is difficult to reach a solid conclusion. In contrast, the analysis of metabolites in patients with CCA, HCC, and non-malignant liver diseases showed a decrease in glycine and taurine-conjugated bile acids, phospholipids, and cholesterol in patients with CCA compared to control groups but only to a certain extent when compared to HCC[84].

In theory, if one biomarker is detected and validated in bile, it might provide sufficient grounds further to test it in more accessible and less invasive fluids (*e.g.*, serum, urine, plasma). Nevertheless, metabolomics studies can also be performed directly on serum, plasma, or urine. Using serum, one study from the United Kingdom failed to show any differences between profiles from patients with benign biliary strictures and CCA[85]. In contrast, one study from China showed that two bile acids, chenodeoxycholic acid (CDCA) and taurochenoxycholic acid (TCDCA) (from plasma), had higher sensitivity and specificity than CA19-9 for CCA *vs* benign bile duct disease and CCA *vs* HC[86]. Furthermore, a study from Europe (Italy), using an artificial intelligence approach, found a plasma bile acid profile that could discriminate between CCA and benign BTD with an accuracy of 86.4%[87]. However, all the beforementioned studies could not offer more answers to some of the most critical clinical dilemmas when caring for patients with liver cancer.

In the liver cancer community, there are at least two primary clinical necessities. The first clinical dilemma is probably the most common scenario: One patient with advanced liver disease and focal liver lesions: Is it cancer? If the answer is yes, is it HCC or iCCA? In this setting, one study (on serum) has shown that the development of an algorithm combining glycine, aspartic acid, sphingomyelin (SM) (42:3), and SM (43:2) permitted accurate discrimination between HCC and iCCA with a sensitivity of 75% and specificity of 90%. In the same study, another algorithm discriminated PSC from iCCA with a sensitivity of 100% and specificity of 70%. Of note, these results were further validated in an

independent cohort[88]. A similar finding was also reported in one study from China. A panel of four metabolites {PE (19:0/0:0), PE [18:2 (9Z, 12Z)/0:0], PC (14:0/0:0) and PC (18:0/0:0)} attained a diagnostic accuracy (HCC *vs* iCCA) of 99.7%[89].

The second clinical dilemma is: One patient with distal bile duct obstruction: Is it cancer? dCCA or pancreatic ductal adenocarcinoma (PDAC)? A combination of serum levels of nine metabolites [acylcarnitine AC (16:0), ceramide Cer (d18:1/24:0), phosphatidylcholines PC (20:0/0:0) and PC (O-16:0/20:3), lysophosphatidylcholines PC (20:0/0:0) and PC (0:0/20:0), lysophosphatidylethanolamine PE (P-18:2/0:0), and sphingomyelins SM (d18:2/22:0) and SM (d18:2/23:0) and CA 19-9] could discriminate between dCCA and PDAC with a sensitivity of 55.9% and specificity of 89.5%[90]. Metabolic profiling of urine in patients with CCA was also applied, showing some metabolic differences in the urine of CCA compared to controls. As such, a urine metabolomic panel consisting of 3-methylhistidine, citric acid, cytosine, indoleacetic acid, salicyluric acid, L-methionine, aminomalonic acid, glutaric acid, ursodeoxycholic acid, N-acetylornithine, allantoin, glycocholic acid, histamine, homogentisic acid, L-kynurenine, sarcosine, pyruvic acid, taurine and methylsuccinic acid were identified as potential biomarkers for primary extrahepatic CCA[91]. Nevertheless, in terms of prognosis, only a few studies have shown the potential of metabolites to predict recurrence or OS[91,92].

The road ahead is still long for metabolomics in CCA. Metabolome studies in CCA have just begun, and some promising metabolites have already been identified. However, a shift from bench to bedside is not expected to appear in the next few years. First, identifying a specific metabolite with diagnostic or prognostic properties is a challenging goal due to the presence of many confounding factors (*e.g.*, age, gender, diet, underlying liver disease, concomitant disease, drugs, and others). Secondly, the results from untargeted metabolomics might be different from targeted metabolomics, according to at least one recent metabolomics study, investigating plasma fetal bile acids towards assessing liver cirrhosis severity[93]. Not least, the reproducibility of many of these studies is a genuine concern (due to multiple analytical platforms, different sample preparation protocols), and standardized procedures are urgently needed.

EXTRACELLULAR VESICLES

In terms of minimally invasive biomarkers, EVs are the "new kids on the market". They hold great promise in the diagnosis and prognosis of cancer. EVs are encountered in all body fluids including blood[94], urine[95] and bile[96]. According to their size and biogenesis there are two classes: (1) Large EVs [also called microvesicles (MVs)] roughly ranging from 100 to 1000 nm in size, which directly bud from the plasma membrane of their parental cell; and (2) Small EVs (also called exosomes) are considerably smaller (below 100 nm) and originate from accumulated intraluminal vesicles within the endomembranous system, forming so-called multivesicular bodies[97].

The function of EVs depends on the type and content (*e.g.*, lipids, proteins, nucleic acids) of their parent cells. They orchestrate many of the processes described by Hanahan *et al*[98] as "Hallmarks of Cancer"[98] either *via* paracrine signaling or horizontal transfer of bioactive agents[99]. In the initial steps of cancer genesis, EVs (released by cancer cells) appear to be responsible for the differentiation of mesenchymal stem cells into fibroblasts, contributing to stroma generation ant thus preparing their tumor niche[100]. Furthermore, EVs could transport miR species from human CCA cells to cancer-associated fibroblasts, a communication between cancer cells and the cancer microenvironment responsible for tumor growth and, later on, CCA cells-derived EVs can transfer oncogenes to normal cholangiocytes, increasing their migration and invasive potential [*via* increasing the expression of beta-catenin (CTNNB1) and decreasing the expression of E-cadherin (CDH1)], hence preparing the final processes of carcinogenesis: Tumor invasion and metastasis[101].

Some studies have already revealed the great potential of EVs content or surface markers in terms of diagnosis. Proteomic profiling of serum EVs has identified a panel of five proteins that could assist CCA diagnosis. The study design included CCA (*n* = 43), PSC (*n* = 30), HCC (*n* = 29) patients and HC (*n* = 32). As such, pantetheinase (VNN1), C-reactive protein, fibrinogen gamma chain (FIBG), immunoglobulin heavy constant alpha 1 (IGHA1) and alpha-1 acid glycoprotein (A1AG1) showed to have an increased concentration in serum EVs of CCA compared to all PSC, HCC and HC. Moreover, a panel of three EVs proteins, namely ficolin-2 (FCN2), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and FIBG showed to be able to discriminate between early-stage CCA and PSC patients with an AUC > 0.88[102].

A major challenge nowadays is the differential diagnosis between HCC and iCCA or between dCCA and PDAC. More often, the final diagnosis (HCC *vs* cCCA) in clinical practice is based on liver biopsy. In terms of EVs surface antigens, one study enrolling 172 patients with liver cancer (HCC or CCA), 54 with cirrhosis and no liver neoplasia, and 202 control subjects, found a combination of tumor-associated microparticles (AnnexinV+ epithelial cell adhesion molecule (EpCAM+) and asialoglycoprotein receptor 1 (ASGPR1+)) could diagnose CCA from healthy individuals and other cancer entities with up to 90% sensitivity. However, it was unable to differentiate between CCA and HCC[103]. Interestingly, later on, the same group in another study including a large set of patients, including 77 CCA, 67 HCC, identified a combination between EVs surface antigens (AnnexinV+ CD44v6, cut-off = 34 number *per* 10^3

AnnexinV+ EVs) together with AFP (cut-off = 30 ng/mL) that could discriminate between HCC and CCA (iCCA and eCCA) with both sensitivity and specificity of 100%[104]. Indeed, it is a novel potential diagnostic biomarker that could help clinicians diagnose CCA non-invasively and accurately. Further large multicenter studies are urgently necessary. In search of novel biomarkers for the differential diagnoses between dCCA and PDAC, one study including 50 patients (*n* = 20 pancreatic cancer, *n* = dCCA, *n* = 15 chronic pancreatitis, *n* = 10 common bile duct obstruction due to biliary stones patients) reported that the concentration of EVs *per se* in bile and serum could discriminate malignant from non-malignant pancreaticobiliary diseases with 100% sensitivity in bile and 47% in serum[105]. EVs cargo profile could also have diagnosis potential. In particular, a panel of 5 miRs (miR-191, miR-486-3p, miR-1274b, miR-16 and miR-484) isolated from bile EVs showed good diagnostic values for CCA diagnosis compared to non☐malignant biliary diseases (sensitivity: 67%; specificity: 96%)[106].

CIRCULATING TUMOR CELLS

Circulating tumor cells (CTCs) have been evaluated as a diagnostic marker in pancreatic, colorectal, breast or prostate cancer, and are associated with poor survival rates. However, only a handful of studies have assessed their potential in CCAs. CTCs are cancer-derived cells released from a primary solid tumor or local lymphoid reservoirs into the bloodstream, harboring tumor-initiation properties, and possibly enabling distant metastasis. Even after primary tumor resection, the permanence of viable CTCs in the portal venous blood seems to be a consequence of T-cell suppression by myeloid-derived suppressor cells and CTC-induced apoptosis[107]. Subsequently, CTCs proliferate and cluster, possibly under the influence of cell adhesion molecules such as plakoglobin, leading to tumor growth and immune resistance[107,108].

Identification of CTCs in peripheral blood relies on their overexpression of EpCAM. It has been performed using immunocytochemistry, reverse transcriptase-PCR, flow cytometry, or an enzyme-linked immunosorbent spot assay. The most used assay is CellSearch™, which uses ferrofluid nanoparticles with antibodies that target EpCAM, which is expressed in various of human epithelial tissues, carcinomas, and stem cells, and is involved in cell signaling, migration, proliferation, and differentiation[109-111]. In a study of 26 CCA patients, targeting CTCs using antibodies against EpCAM, DAPI, cytokeratin 8, 18, and/or 19, Al Ustwani *et al*[112] showed that 25% of patients with CCA had a significant amount of CTCs (≥ 2/7.5 mL of blood). Similar results were reported in another study, where out of 95 CCA patients, 24% had a count of two cells or higher *per* 7.5 mL blood, while 22% had a count of one cell, and the remainder of 54% no detectable cells[113]. Since CTCs seem to be relatively rare in peripheral blood, their potential as a diagnostic marker might be more evident in patients with metastatic disease and less in early tumors.

CTCs are also seemingly associated with more aggressive tumors, as patients with no CTCs in their blood sample had the best survival rate. In contrast, the presence of two or more CTCs was strongly associated with worse OS (median 18.1 mo *vs* 8.7 mo)[113]. However, their presence does not seem to predict treatment outcome, as evidenced in the ABC-03 trial[113,114].

The high degree of variability in detection rates might be explained by suboptimal EpCAM levels for detection, loss of epithelial surface antigens, or epithelial-mesenchymal transition[112,115,116]. To overcome these shortcomings, a novel glycosaminoglycan-SCH45-probe on a microfluidic platform has been employed to isolate CCA CTCs by combining multiple-capture approaches in a shorter period and using lower blood volumes compared to the traditional method. Using EpCAM as a conventional protein biomarker, the authors showed, by analyzing peripheral blood of 65 metastatic CCA patients, that CTCs could be detected in all advanced or metastatic CCA, suggesting that CTCs may maximize the predictive performance of liquid biopsies if the proper diagnostic tool is used[117]. Reduzzi *et al* [118] assessed an alternative to improving detection rates in a prospective study of 21 patients with advanced-stage biliary tract cancer. Using non-conventional CTCs lacking epithelial and leukocyte markers, but presenting aberrant genomes, the detection rate increased from 19% to 83%.

CONCLUSION

A non-invasive approach towards diagnosis and prognosis is the path forward in CCA, a type of cancer that sometimes appears to be hiding in plain sight. The previously discussed methods aim to provide the necessary leap forward towards a personalized approach and might allow for a refined characterization of the disease. However, most available reports are deeply heterogeneous, study protocols are not harmonized, and the number of included patients is inconsistent. These caveats appear to be the primary reasons for the gap between the wide range of cancer biomarkers that appear to be effective in individual studies and the relatively low number of biomarkers ready to be translated into the clinic. Consequently, the most challenging task in the short term might be not to find new molecules and pathways but rather to validate or infirm the role of current methods to shorten the bench to bedside gap.

FOOTNOTES

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REFERENCES

1   **WHO Classification of Tumours Editorial Board**.   Digestive System Tumours, WHO Classification of Tumours. 5th ed. World Health Organization: IARC Publications, 2019, ISBN 978-92-832-4499-8

2   **Tyson GL**, Ilyas JA, Duan Z, Green LK, Younes M, El-Serag HB, Davila JA. Secular trends in the incidence of cholangiocarcinoma in the USA and the impact of misclassification. *Dig Dis Sci* 2014; **59**: 3103-3110 [PMID: 25204668 DOI: 10.1007/s10620-014-3276-2]

3   **Bridgewater JA**, Goodman KA, Kalyan A, Mulcahy MF. Biliary Tract Cancer: Epidemiology, Radiotherapy, and Molecular Profiling. *Am Soc Clin Oncol Educ Book* 2016; **35**: e194-e203 [PMID: 27249723 DOI: 10.1200/EDBK_160831]

4   **Saha SK**, Zhu AX, Fuchs CS, Brooks GA. Forty-Year Trends in Cholangiocarcinoma Incidence in the U.S.: Intrahepatic Disease on the Rise. *Oncologist* 2016; **21**: 594-599 [PMID: 27000463 DOI: 10.1634/theoncologist.2015-0446]

5   **Banales JM**, Cardinale V, Carpino G, Marzioni M, Andersen JB, Invernizzi P, Lind GE, Folseraas T, Forbes SJ, Fouassier L, Geier A, Calvisi DF, Mertens JC, Trauner M, Benedetti A, Maroni L, Vaquero J, Macias RI, Raggi C, Perugorria MJ, Gaudio E, Boberg KM, Marin JJ, Alvaro D. Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). *Nat Rev Gastroenterol Hepatol* 2016; **13**: 261-280 [PMID: 27095655 DOI: 10.1038/nrgastro.2016.51]

6   **Forner A**, Vidili G, Rengo M, Bujanda L, Ponz-Sarvisé M, Lamarca A. Clinical presentation, diagnosis and staging of cholangiocarcinoma. *Liver Int* 2019; **39** Suppl 1: 98-107 [PMID: 30831002 DOI: 10.1111/liv.14086]

7   **Aishima S**, Oda Y. Pathogenesis and classification of intrahepatic cholangiocarcinoma: different characters of perihilar large duct type *vs* peripheral small duct type. *J Hepatobiliary Pancreat Sci* 2015; **22**: 94-100 [PMID: 25181580 DOI: 10.1002/jhbp.154]

8   **Endo I**, Gonen M, Yopp AC, Dalal KM, Zhou Q, Klimstra D, D'Angelica M, DeMatteo RP, Fong Y, Schwartz L, Kemeny N, O'Reilly E, Abou-Alfa GK, Shimada H, Blumgart LH, Jarnagin WR. Intrahepatic cholangiocarcinoma: rising frequency, improved survival, and determinants of outcome after resection. *Ann Surg* 2008; **248**: 84-96 [PMID: 18580211 DOI: 10.1097/SLA.0b013e318176c4d3]

9   **Jiang BG**, Sun LL, Yu WL, Tang ZH, Zong M, Zhang YJ. Retrospective analysis of histopathologic prognostic factors after hepatectomy for intrahepatic cholangiocarcinoma. *Cancer J* 2009; **15**: 257-261 [PMID: 19556914 DOI: 10.1097/PPO.0b013e31819e3312]

10   **DeOliveira ML**, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ, Schulick RD. Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. *Ann Surg* 2007; **245**: 755-762 [PMID: 17457168 DOI: 10.1097/01.sla.0000251366.62632.d3]

11   **Bridgewater J**, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014; **60**: 1268-1289 [PMID: 24681130 DOI: 10.1016/j.jhep.2014.01.021]

12   **Liver Cancer Study Group of Japan**. Primary liver cancer in Japan. Clinicopathologic features and results of surgical

treatment. *Ann Surg* 1990; **211**: 277-287 [PMID: 2155591]

13   **Liang B**, Zhong L, He Q, Wang S, Pan Z, Wang T, Zhao Y. Diagnostic Accuracy of Serum CA19-9 in Patients with Cholangiocarcinoma: A Systematic Review and Meta-Analysis. *Med Sci Monit* 2015; **21**: 3555-3563 [PMID: 26576628 DOI: 10.12659/msm.895040]

14   **Mao ZY**, Guo XC, Su D, Wang LJ, Zhang TT, Bai L. Prognostic Factors of Cholangiocarcinoma After Surgical Resection: A Retrospective Study of 293 Patients. *Med Sci Monit* 2015; **21**: 2375-2381 [PMID: 26269932 DOI: 10.12659/MSM.893586]

15   **Ruys AT**, Groot Koerkamp B, Wiggers JK, Klümpen HJ, ten Kate FJ, van Gulik TM. Prognostic biomarkers in patients with resected cholangiocarcinoma: a systematic review and meta-analysis. *Ann Surg Oncol* 2014; **21**: 487-500 [PMID: 24081803 DOI: 10.1245/s10434-013-3286-x]

16   **Saha SK**, Gordan JD, Kleinstiver BP, Vu P, Najem MS, Yeo JC, Shi L, Kato Y, Levin RS, Webber JT, Damon LJ, Egan RK, Greninger P, McDermott U, Garnett MJ, Jenkins RL, Rieger-Christ KM, Sullivan TB, Hezel AF, Liss AS, Mizukami Y, Goyal L, Ferrone CR, Zhu AX, Joung JK, Shokat KM, Benes CH, Bardeesy N. Isocitrate Dehydrogenase Mutations Confer Dasatinib Hypersensitivity and SRC Dependence in Intrahepatic Cholangiocarcinoma. *Cancer Discov* 2016; **6**: 727-739 [PMID: 27231123 DOI: 10.1158/2159-8290.CD-15-1442]

17   **Xie Q**, Wang L, Zheng S. Prognostic and Clinicopathological Significance of PD-L1 in Patients with Cholangiocarcinoma: A Meta-Analysis. *Dis Markers* 2020; **2020**: 1817931 [PMID: 32724483 DOI: 10.1155/2020/1817931]

18   **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

19   **Macias RIR**, Kornek M, Rodrigues PM, Paiva NA, Castro RE, Urban S, Pereira SP, Cadamuro M, Rupp C, Loosen SH, Luedde T, Banales JM. Diagnostic and prognostic biomarkers in cholangiocarcinoma. *Liver Int* 2019; **39** Suppl 1: 108-122 [PMID: 30843325 DOI: 10.1111/liv.14090]

20   **Shyamala K**, Girish HC, Murgod S. Risk of tumor cell seeding through biopsy and aspiration cytology. *J Int Soc Prev Community Dent* 2014; **4**: 5-11 [PMID: 24818087 DOI: 10.4103/2231-0762.129446]

21   **Leon SA**, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977; **37**: 646-650 [PMID: 837366]

22   **Zviran A**, Schulman RC, Shah M, Hill STK, Deochand S, Khamnei CC, Maloney D, Patel K, Liao W, Widman AJ, Wong P, Callahan MK, Ha G, Reed S, Rotem D, Frederick D, Sharova T, Miao B, Kim T, Gydush G, Rhoades J, Huang KY, Omans ND, Bolan PO, Lipsky AH, Ang C, Malbari M, Spinelli CF, Kazancioglu S, Runnels AM, Fennessey S, Stolte C, Gaiti F, Inghirami GG, Adalsteinsson V, Houck-Loomis B, Ishii J, Wolchok JD, Boland G, Robine N, Altorki NK, Landau DA. Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nat Med* 2020; **26**: 1114-1124 [PMID: 32483360 DOI: 10.1038/s41591-020-0915-3]

23   **Andersen RF**, Jakobsen A. Screening for circulating RAS/RAF mutations by multiplex digital PCR. *Clin Chim Acta* 2016; **458**: 138-143 [PMID: 27181912 DOI: 10.1016/j.cca.2016.05.007]

24   **Ettrich TJ**, Schwerdel D, Dolnik A, Beuter F, Blätte TJ, Schmidt SA, Stanescu-Siegmund N, Steinacker J, Marienfeld R, Kleger A, Bullinger L, Seufferlein T, Berger AW. Genotyping of circulating tumor DNA in cholangiocarcinoma reveals diagnostic and prognostic information. *Sci Rep* 2019; **9**: 13261 [PMID: 31519967 DOI: 10.1038/s41598-019-49860-0]

25   **Goyal L**, Saha SK, Liu LY, Siravegna G, Leshchiner I, Ahronian LG, Lennerz JK, Vu P, Deshpande V, Kambadakone A, Mussolin B, Reyes S, Henderson L, Sun JE, Van Seventer EE, Gurski JM Jr, Baltschukat S, Schacher-Engstler B, Barys L, Stamm C, Furet P, Ryan DP, Stone JR, Iafrate AJ, Getz G, Porta DG, Tiedt R, Bardelli A, Juric D, Corcoran RB, Bardeesy N, Zhu AX. Polyclonal Secondary *FGFR2* Mutations Drive Acquired Resistance to FGFR Inhibition in Patients with FGFR2 Fusion-Positive Cholangiocarcinoma. *Cancer Discov* 2017; **7**: 252-263 [PMID: 28034880 DOI: 10.1158/2159-8290.CD-16-1000]

26   **Wasenang W**, Chaiyarit P, Proungvitaya S, Limpaiboon T. Serum cell-free DNA methylation of OPCML and HOXD9 as a biomarker that may aid in differential diagnosis between cholangiocarcinoma and other biliary diseases. *Clin Epigenetics* 2019; **11**: 39 [PMID: 30832707 DOI: 10.1186/s13148-019-0634-0]

27   **Wang LJ**, He CC, Sui X, Cai MJ, Zhou CY, Ma JL, Wu L, Wang H, Han SX, Zhu Q. MiR-21 promotes intrahepatic cholangiocarcinoma proliferation and growth *in vitro* and *in vivo* by targeting PTPN14 and PTEN. *Oncotarget* 2015; **6**: 5932-5946 [PMID: 25803229 DOI: 10.18632/oncotarget.3465]

28   **Silakit R**, Loilome W, Yongvanit P, Chusorn P, Techasen A, Boonmars T, Khuntikeo N, Chamadol N, Pairojkul C, Namwat N. Circulating miR-192 in liver fluke-associated cholangiocarcinoma patients: a prospective prognostic indicator. *J Hepatobiliary Pancreat Sci* 2014; **21**: 864-872 [PMID: 25131257 DOI: 10.1002/jhbp.145]

29   **Wang LJ**, Zhang KL, Zhang N, Ma XW, Yan SW, Cao DH, Shi SJ. Serum miR-26a as a diagnostic and prognostic biomarker in cholangiocarcinoma. *Oncotarget* 2015; **6**: 18631-18640 [PMID: 26087181 DOI: 10.18632/oncotarget.4072]

30   **Hu JJ**, Zhou C, Luo X, Luo SZ, Li ZH, Xu ZX, Xu MY. Linc-SCRG1 accelerates progression of hepatocellular carcinoma as a ceRNA of miR26a to derepress SKP2. *J Exp Clin Cancer Res* 2021; **40**: 26 [PMID: 33422101 DOI: 10.1186/s13046-020-01825-2]

31   **Han HS**, Kim MJ, Han JH, Yun J, Kim HK, Yang Y, Kim KB, Park SM. Bile-derived circulating extracellular miR-30d-5p and miR-92a-3p as potential biomarkers for cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2020; **19**: 41-50 [PMID: 31784323 DOI: 10.1016/j.hbpd.2019.10.009]

32   **Soliman SE**, Elabd NS, El-Kousy SM, Awad MF. Down regulation of miR-30a-5p and miR-182-5p in gastric cancer: Clinical impact and survival analysis. *Biochem Biophys Rep* 2021; **27**: 101079 [PMID: 34355069 DOI: 10.1016/j.bbrep.2021.101079]

33   **Bernuzzi F**, Marabita F, Lleo A, Carbone M, Mirolo M, Marzioni M, Alpini G, Alvaro D, Boberg KM, Locati M, Torzilli G, Rimassa L, Piscaglia F, He XS, Bowlus CL, Yang GX, Gershwin ME, Invernizzi P. Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Clin Exp Immunol* 2016; **185**: 61-71 [PMID:

26864161 DOI: 10.1111/cei.12776]

34   **Correa-Gallego C**, Maddalo D, Doussot A, Kemeny N, Kingham TP, Allen PJ, D'Angelica MI, DeMatteo RP, Betel D, Klimstra D, Jarnagin WR, Ventura A. Circulating Plasma Levels of MicroRNA-21 and MicroRNA-221 Are Potential Diagnostic Markers for Primary Intrahepatic Cholangiocarcinoma. *PLoS One* 2016; **11**: e0163699 [PMID: 27685844 DOI: 10.1371/journal.pone.0163699]

35   **Silakit R**, Loilome W, Yongvanit P, Thongchot S, Sithithaworn P, Boonmars T, Koonmee S, Titapun A, Khuntikeo N, Chamadol N, Techasen A, Namwat N. Urinary microRNA-192 and microRNA-21 as potential indicators for liver fluke-associated cholangiocarcinoma risk group. *Parasitol Int* 2017; **66**: 479-485 [PMID: 26456596 DOI: 10.1016/j.parint.2015.10.001]

36   **Xu J**, Wu C, Che X, Wang L, Yu D, Zhang T, Huang L, Li H, Tan W, Wang C, Lin D. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog* 2011; **50**: 136-142 [PMID: 21229610 DOI: 10.1002/mc.20712]

37   **Goto T**, Fujiya M, Konishi H, Sasajima J, Fujibayashi S, Hayashi A, Utsumi T, Sato H, Iwama T, Ijiri M, Sakatani A, Tanaka K, Nomura Y, Ueno N, Kashima S, Moriichi K, Mizukami Y, Kohgo Y, Okumura T. An elevated expression of serum exosomal microRNA-191, - 21, -451a of pancreatic neoplasm is considered to be efficient diagnostic marker. *BMC Cancer* 2018; **18**: 116 [PMID: 29385987 DOI: 10.1186/s12885-018-4006-5]

38   **Sierzega M**, Kaczor M, Kolodziejczyk P, Kulig J, Sanak M, Richter P. Evaluation of serum microRNA biomarkers for gastric cancer based on blood and tissue pools profiling: the importance of miR-21 and miR-331. *Br J Cancer* 2017; **117**: 266-273 [PMID: 28641313 DOI: 10.1038/bjc.2017.190]

39   **Liu CH**, Huang Q, Jin ZY, Xie F, Zhu CL, Liu Z, Wang C. Circulating microRNA-21 as a prognostic, biological marker in cholangiocarcinoma. *J Cancer Res Ther* 2018; **14**: 220-225 [PMID: 29516989 DOI: 10.4103/0973-1482.193125]

40   **Wu X**, Xia M, Chen D, Wu F, Lv Z, Zhan Q, Jiao Y, Wang W, Chen G, An F. Profiling of downregulated blood-circulating miR-150-5p as a novel tumor marker for cholangiocarcinoma. *Tumour Biol* 2016; **37**: 15019-15029 [PMID: 27658773 DOI: 10.1007/s13277-016-5313-6]

41   **Chen X**, Xu X, Pan B, Zeng K, Xu M, Liu X, He B, Pan Y, Sun H, Wang S. Correction for: miR-150-5p suppresses tumor progression by targeting VEGFA in colorectal cancer. *Aging (Albany NY)* 2021; **13**: 13372-13373 [PMID: 33988523 DOI: 10.18632/aging.203069]

42   **Zhou J**, Liu Z, Yang S, Li X. Identification of microRNAs as biomarkers for cholangiocarcinoma detection: A diagnostic meta-analysis. *Clin Res Hepatol Gastroenterol* 2017; **41**: 156-162 [PMID: 27939910 DOI: 10.1016/j.clinre.2016.10.007]

43   **Liang Z**, Liu X, Zhang Q, Wang C, Zhao Y. Diagnostic value of microRNAs as biomarkers for cholangiocarcinoma. *Dig Liver Dis* 2016; **48**: 1227-1232 [PMID: 27476468 DOI: 10.1016/j.dld.2016.07.006]

44   **Plieskatt J**, Rinaldi G, Feng Y, Peng J, Easley S, Jia X, Potriquet J, Pairojkul C, Bhudhisawasdi V, Sripa B, Brindley PJ, Bethony J, Mulvenna J. A microRNA profile associated with Opisthorchis viverrini-induced cholangiocarcinoma in tissue and plasma. *BMC Cancer* 2015; **15**: 309 [PMID: 25903557 DOI: 10.1186/s12885-015-1270-5]

45   **Lang SA**, Bednarsch J, Joechle K, Amygdalos I, Czigany Z, Heij L, Ulmer TF, Neumann UP. Prognostic biomarkers for cholangiocarcinoma (CCA): state of the art. *Expert Rev Gastroenterol Hepatol* 2021; **15**: 497-510 [PMID: 33970740 DOI: 10.1080/17474124.2021.1912591]

46   **Rompianesi G**, Di Martino M, Gordon-Weeks A, Montalti R, Troisi R. Liquid biopsy in cholangiocarcinoma: Current status and future perspectives. *World J Gastrointest Oncol* 2021; **13**: 332-350 [PMID: 34040697 DOI: 10.4251/wjgo.v13.i5.332]

47   **Tsen A**, Barbara M, Rosenkranz L. Dilemma of elevated CA 19-9 in biliary pathology. *Pancreatology* 2018; **18**: 862-867 [PMID: 30249386 DOI: 10.1016/j.pan.2018.09.004]

48   **Zhang M**, Cheng S, Jin Y, Zhao Y, Wang Y. Roles of CA125 in diagnosis, prediction, and oncogenesis of ovarian cancer. *Biochim Biophys Acta Rev Cancer* 2021; **1875**: 188503 [PMID: 33421585 DOI: 10.1016/j.bbcan.2021.188503]

49   **You YN**, Hardiman KM, Bafford A, Poylin V, Francone TD, Davis K, Paquette IM, Steele SR, Feingold DL; On Behalf of the Clinical Practice Guidelines Committee of the American Society of Colon and Rectal Surgeons. The American Society of Colon and Rectal Surgeons Clinical Practice Guidelines for the Management of Rectal Cancer. *Dis Colon Rectum* 2020; **63**: 1191-1222 [PMID: 33216491 DOI: 10.1097/DCR.0000000000001762]

50   **Loosen SH**, Roderburg C, Kauertz KL, Pombeiro I, Leyh C, Benz F, Vucur M, Longerich T, Koch A, Braunschweig T, Ulmer TF, Heidenhain C, Tacke F, Binnebösel M, Schmeding M, Trautwein C, Neumann UP, Luedde T. Elevated levels of circulating osteopontin are associated with a poor survival after resection of cholangiocarcinoma. *J Hepatol* 2017; **67**: 749-757 [PMID: 28668580 DOI: 10.1016/j.jhep.2017.06.020]

51   **Onsurathum S**, Haonon O, Pinlaor P, Pairojkul C, Khuntikeo N, Thanan R, Roytrakul S, Pinlaor S. Proteomics detection of S100A6 in tumor tissue interstitial fluid and evaluation of its potential as a biomarker of cholangiocarcinoma. *Tumour Biol* 2018; **40**: 1010428318767195 [PMID: 29629840 DOI: 10.1177/1010428318767195]

52   **Shi RY**, Yang XR, Shen QJ, Yang LX, Xu Y, Qiu SJ, Sun YF, Zhang X, Wang Z, Zhu K, Qin WX, Tang ZY, Fan J, Zhou J. High expression of Dickkopf-related protein 1 is related to lymphatic metastasis and indicates poor prognosis in intrahepatic cholangiocarcinoma patients after surgery. *Cancer* 2013; **119**: 993-1003 [PMID: 23132676 DOI: 10.1002/cncr.27788]

53   **Cheon YK**, Cho YD, Moon JH, Jang JY, Kim YS, Lee MS, Lee JS, Shim CS. Diagnostic utility of interleukin-6 (IL-6) for primary bile duct cancer and changes in serum IL-6 Levels following photodynamic therapy. *Am J Gastroenterol* 2007; **102**: 2164-2170 [PMID: 17617204 DOI: 10.1111/j.1572-0241.2007.01403.x]

54   **Shen J**, Wang W, Wu J, Feng B, Chen W, Wang M, Tang J, Wang F, Cheng F, Pu L, Tang Q, Wang X, Li X. Comparative proteomic profiling of human bile reveals SSP411 as a novel biomarker of cholangiocarcinoma. *PLoS One* 2012; **7**: e47476 [PMID: 23118872 DOI: 10.1371/journal.pone.0047476]

55   **Ruzzenente A**, Iacono C, Conci S, Bertuzzo F, Salvagno G, Ruzzenente O, Campagnaro T, Valdegamberi A, Pachera S, Bagante F, Guglielmi A. A novel serum marker for biliary tract cancer: diagnostic and prognostic values of quantitative evaluation of serum mucin 5AC (MUC5AC). *Surgery* 2014; **155**: 633-639 [PMID: 24468034 DOI: 10.1016/j.surg.2013.12.003]

56   **Leelawat K**, Sakchinabut S, Narong S, Wannaprasert J. Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: evaluation of diagnostic accuracy. *BMC Gastroenterol* 2009; **9**: 30 [PMID: 19405942 DOI: 10.1186/1471-230X-9-30]

57   **Leelawat K**, Narong S, Wannaprasert J, Ratanashu-ek T. Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma. *World J Gastroenterol* 2010; **16**: 4697-4703 [PMID: 20872971 DOI: 10.3748/wjg.v16.i37.4697]

58   **Guowei H**, Yuan L, Ma L, Zhongyang L, Zhixing S, Lin L, Minqi L. The diagnostic efficacy of CYFRA21-1 on intrahepatic cholangiocarcinoma: A meta-analysis. *Clin Res Hepatol Gastroenterol* 2019; **43**: 266-272 [PMID: 30503663 DOI: 10.1016/j.clinre.2018.10.010]

59   **Loosen SH**, Breuer A, Tacke F, Kather JN, Gorgulho J, Alizai PH, Bednarsch J, Roeth AA, Lurje G, Schmitz SM, Brozat JF, Paffenholz P, Vucur M, Ritz T, Koch A, Trautwein C, Ulmer TF, Roderburg C, Longerich T, Neumann UP, Luedde T. Circulating levels of soluble urokinase plasminogen activator receptor predict outcome after resection of biliary tract cancer. *JHEP Rep* 2020; **2**: 100080 [PMID: 32140677 DOI: 10.1016/j.jhepr.2020.100080]

60   **Kim SY**, Lee HS, Bang SM, Han DH, Hwang HK, Choi GH, Chung MJ, Kim SU. Serum Dickkopf-1 in Combined with CA 19-9 as a Biomarker of Intrahepatic Cholangiocarcinoma. *Cancers (Basel)* 2021; **13** [PMID: 33921232 DOI: 10.3390/cancers13081828]

61   **Byrling J**, Hilmersson KS, Ansari D, Andersson R, Andersson B. Thrombospondin-2 as a diagnostic biomarker for distal cholangiocarcinoma and pancreatic ductal adenocarcinoma. *Clin Transl Oncol* 2022; **24**: 297-304 [PMID: 34319497 DOI: 10.1007/s12094-021-02685-8]

62   **Kimawaha P**, Jusakul A, Junsawang P, Thanan R, Titapun A, Khuntikeo N, Techasen A. Establishment of a Potential Serum Biomarker Panel for the Diagnosis and Prognosis of Cholangiocarcinoma Using Decision Tree Algorithms. *Diagnostics (Basel)* 2021; **11** [PMID: 33806004 DOI: 10.3390/diagnostics11040589]

63   **Xu HL**, Inagaki Y, Seyama Y, Sugawara Y, Kokudo N, Nakata M, Wang FS, Tang W. Expression of KL-6 mucin, a human MUC1 mucin, in intrahepatic cholangiocarcinoma and its potential involvement in tumor cell adhesion and invasion. *Life Sci* 2009; **85**: 395-400 [PMID: 19631667 DOI: 10.1016/j.lfs.2009.07.004]

64   **Zheng BH**, Shen S, Wong KF, Gong ZJ, Sun WT, Ni XJ, Wang JW, Hu MY, Liu H, Ni XL, Liu HB, Luk JM, Suo T. Clinical correlation of cadherin-17 marker with advanced tumor stages and poor prognosis of cholangiocarcinoma. *J Surg Oncol* 2021; **123**: 1253-1262 [PMID: 33524213 DOI: 10.1002/jso.26399]

65   **Siriphak S**, Chanakankun R, Proungvitaya T, Roytrakul S, Tummanatsakun D, Seubwai W, Wongwattanakul M, Proungvitaya S. Kallikrein-11, in Association with Coiled-Coil Domain Containing 25, as a Potential Prognostic Marker for Cholangiocarcinoma with Lymph Node Metastasis. *Molecules* 2021; **26** [PMID: 34067437 DOI: 10.3390/molecules26113105]

66   **Truong SDA**, Tummanatsakun D, Proungvitaya T, Limpaiboon T, Wongwattanakul M, Chua-On D, Roytrakul S, Proungvitaya S. Serum Levels of Cytokine-Induced Apoptosis Inhibitor 1 (CIAPIN1) as a Potential Prognostic Biomarker of Cholangiocarcinoma. *Diagnostics (Basel)* 2021; **11** [PMID: 34201138 DOI: 10.3390/diagnostics11061054]

67   **Loosen SH**, Benz F, Niedeggen J, Schmeding M, Schüller F, Koch A, Vucur M, Tacke F, Trautwein C, Roderburg C, Neumann UP, Luedde T. Serum levels of S100A6 are unaltered in patients with resectable cholangiocarcinoma. *Clin Transl Med* 2016; **5**: 39 [PMID: 27709523 DOI: 10.1186/s40169-016-0120-7]

68   **Grunnet M**, Christensen IJ, Lassen U, Jensen LH, Lydolph M, Lund IK, Thurison T, Høyer-Hansen G, Mau-Sørensen M. Prognostic significance of circulating intact and cleaved forms of urokinase plasminogen activator receptor in inoperable chemotherapy treated cholangiocarcinoma patients. *Clin Biochem* 2014; **47**: 599-604 [PMID: 24530340 DOI: 10.1016/j.clinbiochem.2014.01.030]

69   **Liu BX**, Tang CT, Dai XJ, Zeng L, Cheng F, Chen Y, Zeng C. Prognostic Value of S100P Expression in Patients With Digestive System Cancers: A Meta-Analysis. *Front Oncol* 2021; **11**: 593728 [PMID: 33747914 DOI: 10.3389/fonc.2021.593728]

70   **Perng DS**, Hung CM, Lin HY, Morgan P, Hsu YC, Wu TC, Hsieh PM, Yeh JH, Hsiao P, Lee CY, Li YC, Wang YC, Chen YS, Lin CW. Role of autophagy-related protein in the prognosis of combined hepatocellular carcinoma and cholangiocarcinoma after surgical resection. *BMC Cancer* 2021; **21**: 828 [PMID: 34273969 DOI: 10.1186/s12885-021-08553-6]

71   **Thummarati P**, Wijitburaphat S, Prasopthum A, Menakongka A, Sripa B, Tohtong R, Suthiphongchai T. High level of urokinase plasminogen activator contributes to cholangiocarcinoma invasion and metastasis. *World J Gastroenterol* 2012; **18**: 244-250 [PMID: 22294827 DOI: 10.3748/wjg.v18.i3.244]

72   **Itatsu K**, Zen Y, Yamaguchi J, Ohira S, Ishikawa A, Ikeda H, Sato Y, Harada K, Sasaki M, Sakamoto H, Nagino M, Nimura Y, Ohta T, Nakanuma Y. Expression of matrix metalloproteinase 7 is an unfavorable postoperative prognostic factor in cholangiocarcinoma of the perihilar, hilar, and extrahepatic bile ducts. *Hum Pathol* 2008; **39**: 710-719 [PMID: 18329694 DOI: 10.1016/j.humpath.2007.09.016]

73   **Hirashita T**, Iwashita Y, Ohta M, Komori Y, Eguchi H, Yada K, Kitano S. Expression of matrix metalloproteinase-7 is an unfavorable prognostic factor in intrahepatic cholangiocarcinoma. *J Gastrointest Surg* 2012; **16**: 842-848 [PMID: 22246855 DOI: 10.1007/s11605-011-1813-2]

74   **Zhang C**, Xu J, Ye J, Zhang X. Prognostic value of HHLA2 expression in solid tumors: A meta-analysis based on the Chinese population. *Medicine (Baltimore)* 2021; **100**: e26789 [PMID: 34397730 DOI: 10.1097/MD.0000000000026789]

75   **Qiang Z**, Zhang W, Jin S, Dai K, He Y, Tao L, Yu H. Carcinoembryonic antigen, α-fetoprotein, and Ki67 as biomarkers and prognostic factors in intrahepatic cholangiocarcinoma: A retrospective cohort study. *Ann Hepatol* 2021; **20**: 100242 [PMID: 32841741 DOI: 10.1016/j.aohep.2020.07.010]

76   **Roderburg C**, Loosen SH, Bednarsch J, Alizai PH, Roeth AA, Schmitz SM, Vucur M, Luedde M, Paffenholz P, Tacke F, Trautwein C, Ulmer TF, Neumann UP, Luedde T. Levels of Circulating PD-L1 Are Decreased in Patients with Resectable Cholangiocarcinoma. *Int J Mol Sci* 2021; **22** [PMID: 34207359 DOI: 10.3390/ijms22126569]

77   **Zhou KQ**, Liu WF, Yang LX, Sun YF, Hu J, Chen FY, Zhou C, Zhang XY, Peng YF, Yu L, Zhou J, Fan J, Wang Z. Circulating osteopontin per tumor volume as a prognostic biomarker for resectable intrahepatic cholangiocarcinoma. *Hepatobiliary Surg Nutr* 2019; **8**: 582-596 [PMID: 31929985 DOI: 10.21037/hbsn.2019.03.14]

78   **Kimawaha P**, Jusakul A, Junsawang P, Loilome W, Khuntikeo N, Techasen A. Circulating TGF-β1 as the potential epithelial mesenchymal transition-biomarker for diagnosis of cholangiocarcinoma. *J Gastrointest Oncol* 2020; **11**: 304-318 [PMID: 32399272 DOI: 10.21037/jgo.2019.01.03]

79   **Boroughs LK**, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol* 2015; **17**: 351-359 [PMID: 25774832 DOI: 10.1038/ncb3124]

80   **Macias RIR**, Banales JM, Sangro B, Muntané J, Avila MA, Lozano E, Perugorria MJ, Padillo FJ, Bujanda L, Marin JJG. The search for novel diagnostic and prognostic biomarkers in cholangiocarcinoma. *Biochim Biophys Acta Mol Basis Dis* 2018; **1864**: 1468-1477 [PMID: 28782657 DOI: 10.1016/j.bbadis.2017.08.002]

81   **Park JY**, Park BK, Ko JS, Bang S, Song SY, Chung JB. Bile acid analysis in biliary tract cancer. *Yonsei Med J* 2006; **47**: 817-825 [PMID: 17191311 DOI: 10.3349/ymj.2006.47.6.817]

82   **Albiin N**, Smith IC, Arnelo U, Lindberg B, Bergquist A, Dolenko B, Bryksina N, Bezabeh T. Detection of cholangiocarcinoma with magnetic resonance spectroscopy of bile in patients with and without primary sclerosing cholangitis. *Acta Radiol* 2008; **49**: 855-862 [PMID: 18608012 DOI: 10.1080/02841850802220092]

83   **Sharif AW**, Williams HR, Lampejo T, Khan SA, Bansi DS, Westaby D, Thillainayagam AV, Thomas HC, Cox IJ, Taylor-Robinson SD. Metabolic profiling of bile in cholangiocarcinoma using *in vitro* magnetic resonance spectroscopy. *HPB (Oxford)* 2010; **12**: 396-402 [PMID: 20662790 DOI: 10.1111/j.1477-2574.2010.00185.x]

84   **Nagana Gowda GA**, Shanaiah N, Cooper A, Maluccio M, Raftery D. Visualization of bile homeostasis using (1)H-NMR spectroscopy as a route for assessing liver cancer. *Lipids* 2009; **44**: 27-35 [PMID: 18982376 DOI: 10.1007/s11745-008-3254-6]

85   **Alsaleh M**, Leftley Z, Barbera TA, Koomson LK, Zabron A, Crossey MME, Reeves HL, Cramp M, Ryder S, Greer S, Prince M, Sithithaworn P, Shariff M, Khuntikeo N, Loilome W, Yongvanit P, Shen YL, Cox IJ, Williams R, Wadsworth CA, Holmes E, Nash K, Taylor-Robinson SD. Characterisation of the Serum Metabolic Signature of Cholangiocarcinoma in a United Kingdom Cohort. *J Clin Exp Hepatol* 2020; **10**: 17-29 [PMID: 32025163 DOI: 10.1016/j.jceh.2019.06.001]

86   **Zhang X**, Yang Z, Shi Z, Zhu Z, Li C, Du Z, Zhang Y, Wang Z, Jiao Z, Tian X, Zhang J, Zhai W, Kan Q. Analysis of bile acid profile in plasma to differentiate cholangiocarcinoma from benign biliary diseases and healthy controls. *J Steroid Biochem Mol Biol* 2021; **205**: 105775 [PMID: 33130021 DOI: 10.1016/j.jsbmb.2020.105775]

87   **Negrini D**, Zecchin P, Ruzzenente A, Bagante F, De Nitto S, Gelati M, Salvagno GL, Danese E, Lippi G. Machine Learning Model Comparison in the Screening of Cholangiocarcinoma Using Plasma Bile Acids Profiles. *Diagnostics (Basel)* 2020; **10** [PMID: 32748848 DOI: 10.3390/diagnostics10080551]

88   **Banales JM**, Iñarrairaegui M, Arbelaiz A, Milkiewicz P, Muntané J, Muñoz-Bellvis L, La Casta A, Gonzalez LM, Arretxe E, Alonso C, Martínez-Arranz I, Lapitz A, Santos-Laso A, Avila MA, Martínez-Chantar ML, Bujanda L, Marin JJG, Sangro B, Macias RIR. Serum Metabolites as Diagnostic Biomarkers for Cholangiocarcinoma, Hepatocellular Carcinoma, and Primary Sclerosing Cholangitis. *Hepatology* 2019; **70**: 547-562 [PMID: 30325540 DOI: 10.1002/hep.30319]

89   **Liang Q**, Wang C, Li B, Zhang AH. Lipidomics analysis based on liquid chromatography mass spectrometry for hepatocellular carcinoma and intrahepatic cholangiocarcinoma. *RSC Adv* 2015; **5**: 63711-63718 [DOI: 10.1039/c5ra09589a]

90   **Macias RIR**, Muñoz-Bellvís L, Sánchez-Martín A, Arretxe E, Martínez-Arranz I, Lapitz A, Gutiérrez ML, La Casta A, Alonso C, González LM, Avila MA, Martinez-Chantar ML, Castro RE, Bujanda L, Banales JM, Marin JJG. A Novel Serum Metabolomic Profile for the Differential Diagnosis of Distal Cholangiocarcinoma and Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* 2020; **12** [PMID: 32486461 DOI: 10.3390/cancers12061433]

91   **Wang X**, Li J, Zhang AH. Urine metabolic phenotypes analysis of extrahepatic cholangiocarcinoma disease using ultra-high performance liquid chromatography-mass spectrometry. *RSC Adv* 2016; **6**: 63049-63057 [DOI: 10.1039/c6ra09430a]

92   **Alsaleh M**, Barbera TA, Reeves HL, Cramp ME, Ryder S, Gabra H, Nash K, Shen YL, Holmes E, Williams R, Taylor-Robinson SD. Characterization of the urinary metabolic profile of cholangiocarcinoma in a United Kingdom population. *Hepat Med* 2019; **11**: 47-67 [PMID: 31118840 DOI: 10.2147/HMER.S193996]

93   **Mocan T**, Kang DW, Molloy BJ, Jeon H, Spârchez ZA, Beyoğlu D, Idle JR. Plasma fetal bile acids 7α-hydroxy-3-oxochol-4-en-24-oic acid and 3-oxachola-4,6-dien-24-oic acid indicate severity of liver cirrhosis. *Sci Rep* 2021; **11**: 8298 [PMID: 33859329 DOI: 10.1038/s41598-021-87921-5]

94   **Caby MP**, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol* 2005; **17**: 879-887 [PMID: 15908444 DOI: 10.1093/intimm/dxh267]

95   **Pisitkun T**, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004; **101**: 13368-13373 [PMID: 15326289 DOI: 10.1073/pnas.0403453101]

96   **Masyuk AI**, Huang BQ, Ward CJ, Gradilone SA, Banales JM, Masyuk TV, Radtke B, Splinter PL, LaRusso NF. Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia. *Am J Physiol Gastrointest Liver Physiol* 2010; **299**: G990-G999 [PMID: 20634433 DOI: 10.1152/ajpgi.00093.2010]

97   **Théry C**, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D,

Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, Kovács ÁF, Krämer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B, Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; **7**: 1535750 [PMID: 30637094 DOI: 10.1080/20013078.2018.1535750]

98   **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

99   **Slomka A**, Mocan T, Wang B, Nenu I, Urban SK, Gonzales-Carmona M, Schmidt-Wolf IGH, Lukacs-Kornek V, Strassburg CP, Spârchez Z, Kornek M. EVs as Potential New Therapeutic Tool/Target in Gastrointestinal Cancer and HCC. *Cancers (Basel)* 2020; **12** [PMID: 33080904 DOI: 10.3390/cancers12103019]

100   **Dutta S**, Reamtong O, Panvongsa W, Kitdumrongthum S, Janpipatkul K, Sangvanich P, Piyachaturawat P, Chairoungdua A. Proteomics profiling of cholangiocarcinoma exosomes: A potential role of oncogenic protein transferring in cancer progression. *Biochim Biophys Acta* 2015; **1852**: 1989-1999 [PMID: 26148937 DOI: 10.1016/j.bbadis.2015.06.024]

101   **Haga H**, Yan IK, Takahashi K, Wood J, Zubair A, Patel T. Tumour cell-derived extracellular vesicles interact with mesenchymal stem cells to modulate the microenvironment and enhance cholangiocarcinoma growth. *J Extracell Vesicles* 2015; **4**: 24900 [PMID: 25557794 DOI: 10.3402/jev.v4.24900]

102   **Arbelaiz A**, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, Erice O, Gonzalez E, Jimenez-Agüero R, Lacasta A, Ibarra C, Sanchez-Campos A, Jimeno JP, Lammert F, Milkiewicz P, Marzioni M, Macias RIR, Marin JJG, Patel T, Gores GJ, Martinez I, Elortza F, Falcon-Perez JM, Bujanda L, Banales JM. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Hepatology* 2017; **66**: 1125-1143 [PMID: 28555885 DOI: 10.1002/hep.29291]

103   **Julich-Haertel H**, Urban SK, Krawczyk M, Willms A, Jankowski K, Patkowski W, Kruk B, Krasnodębski M, Ligocka J, Schwab R, Richardsen I, Schaaf S, Klein A, Gehlert S, Sänger H, Casper M, Banales JM, Schuppan D, Milkiewicz P, Lammert F, Lukacs-Kornek V, Kornek M. Cancer-associated circulating large extracellular vesicles in cholangiocarcinoma and hepatocellular carcinoma. *J Hepatol* 2017; **67**: 282-292 [PMID: 28267620 DOI: 10.1016/j.jhep.2017.02.024]

104   **Urban SK**, Sänger H, Krawczyk M, Julich-Haertel H, Willms A, Ligocka J, Azkargorta M, Mocan T, Kahlert C, Kruk B, Jankowski K, Patkowski W, Zieniewicz K, Hołówko W, Krupa Ł, Rzucidło M, Gutkowski K, Wystrychowski W, Król R, Raszeja-Wyszomirska J, Słomka A, Schwab R, Wöhler A, Gonzalez-Carmona MA, Gehlert S, Sparchez Z, Banales JM, Strassburg CP, Lammert F, Milkiewicz P, Kornek M. Synergistic effects of extracellular vesicle phenotyping and AFP in hepatobiliary cancer differentiation. *Liver Int* 2020; **40**: 3103-3116 [PMID: 32614460 DOI: 10.1111/liv.14585]

105   **Severino V**, Dumonceau JM, Delhaye M, Moll S, Annessi-Ramseyer I, Robin X, Frossard JL, Farina A. Extracellular Vesicles in Bile as Markers of Malignant Biliary Stenoses. *Gastroenterology* 2017; **153**: 495-504.e8 [PMID: 28479376 DOI: 10.1053/j.gastro.2017.04.043]

106   **Li L**, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, Georgiades C, Singh VK, Khashab M, Amateau S, Li Z, Okolo P, Lennon AM, Saxena P, Geschwind JF, Schlachter T, Hong K, Pawlik TM, Canto M, Law J, Sharaiha R, Weiss CR, Thuluvath P, Goggins M, Shin EJ, Peng H, Kumbhari V, Hutfless S, Zhou L, Mezey E, Meltzer SJ, Karchin R, Selaru FM. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology* 2014; **60**: 896-907 [PMID: 24497320 DOI: 10.1002/hep.27050]

107   **Arnoletti JP**, Fanaian N, Reza J, Sause R, Almodovar AJ, Srivastava M, Patel S, Veldhuis PP, Griffith E, Shao YP, Zhu X, Litherland SA. Pancreatic and bile duct cancer circulating tumor cells (CTC) form immune-resistant multi-cell type clusters in the portal venous circulation. *Cancer Biol Ther* 2018; **19**: 887-897 [PMID: 30067440 DOI: 10.1080/15384047.2018.1480292]

108   **Goto W**, Kashiwagi S, Asano Y, Takada K, Takahashi K, Hatano T, Takashima T, Tomita S, Motomura H, Ohsawa M,

Hirakawa K, Ohira M. Circulating tumor cell clusters-associated gene plakoglobin is a significant prognostic predictor in patients with breast cancer. *Biomark Res* 2017; **5**: 19 [PMID: 28507762 DOI: 10.1186/s40364-017-0099-2]

109   **Osta WA**, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, Cole DJ, Gillanders WE. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. *Cancer Res* 2004; **64**: 5818-5824 [PMID: 15313925 DOI: 10.1158/0008-5472.CAN-04-0754]

110   **Maetzel D**, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O. Nuclear signalling by tumour-associated antigen EpCAM. *Nat Cell Biol* 2009; **11**: 162-171 [PMID: 19136966 DOI: 10.1038/ncb1824]

111   **Litvinov SV**, van Driel W, van Rhijn CM, Bakker HA, van Krieken H, Fleuren GJ, Warnaar SO. Expression of Ep-CAM in cervical squamous epithelia correlates with an increased proliferation and the disappearance of markers for terminal differentiation. *Am J Pathol* 1996; **148**: 865-875 [PMID: 8774141]

112   **Al Ustwani O**, Iancu D, Yacoub R, Iyer R. Detection of circulating tumor cells in cancers of biliary origin. *J Gastrointest Oncol* 2012; **3**: 97-104 [PMID: 22811877 DOI: 10.3978/j.issn.2078-6891.2011.047]

113   **Valle JW**, Wasan H, Lopes A, Backen AC, Palmer DH, Morris K, Duggan M, Cunningham D, Anthoney DA, Corrie P, Madhusudan S, Maraveyas A, Ross PJ, Waters JS, Steward WP, Rees C, Beare S, Dive C, Bridgewater JA. Cediranib or placebo in combination with cisplatin and gemcitabine chemotherapy for patients with advanced biliary tract cancer (ABC-03): a randomised phase 2 trial. *Lancet Oncol* 2015; **16**: 967-978 [PMID: 26179201 DOI: 10.1016/S1470-2045(15)00139-4]

114   **Backen AC**, Lopes A, Wasan H, Palmer DH, Duggan M, Cunningham D, Anthoney A, Corrie PG, Madhusudan S, Maraveyas A, Ross PJ, Waters JS, Steward WP, Rees C, McNamara MG, Beare S, Bridgewater JA, Dive C, Valle JW. Circulating biomarkers during treatment in patients with advanced biliary tract cancer receiving cediranib in the UK ABC-03 trial. *Br J Cancer* 2018; **119**: 27-35 [PMID: 29925934 DOI: 10.1038/s41416-018-0132-8]

115   **Sieuwerts AM**, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, Martens JW, Gratama JW, Sleijfer S, Foekens JA. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst* 2009; **101**: 61-66 [PMID: 19116383 DOI: 10.1093/jnci/djn419]

116   **Budd GT**, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, Matera J, Repollet M, Doyle GV, Terstappen LW, Hayes DF. Circulating tumor cells *vs* imaging--predicting overall survival in metastatic breast cancer. *Clin Cancer Res* 2006; **12**: 6403-6409 [PMID: 17085652 DOI: 10.1158/1078-0432.CCR-05-1769]

117   **Gopinathan P**, Chiang NJ, Bandaru A, Sinha A, Huang WY, Hung SC, Shan YS, Lee GB. Exploring Circulating Tumor Cells in Cholangiocarcinoma Using a Novel Glycosaminoglycan Probe on a Microfluidic Platform. *Adv Healthc Mater* 2020; **9**: e1901875 [PMID: 32329247 DOI: 10.1002/adhm.201901875]

118   **Reduzzi C**, Vismara M, Silvestri M, Celio L, Niger M, Peverelli G, De Braud F, Daidone MG, Cappelletti V. A novel circulating tumor cell subpopulation for treatment monitoring and molecular characterization in biliary tract cancer. *Int J Cancer* 2020; **146**: 3495-3503 [PMID: 31814120 DOI: 10.1002/ijc.32822]

MINIREVIEWS

COVID-19 and liver dysfunction: What nutritionists need to know

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 has brought serious challenges for the medical field. Patients with COVID-19 usually have respiratory symptoms. However, liver dysfunction is not an uncommon presentation. Additionally, the degree of liver dysfunction is associated with the severity and prognosis of COVID-19. Prevention, diagnosis, and treatment of malnutrition should be routinely recommended in the management of patients with COVID-19, especially in those with liver dysfunction. Recently, a large number of studies have reported that nutrition therapy measures, including natural dietary supplements, vitamins, minerals and trace elements, and probiotics, might have potential hepatoprotective effects against COVID-19-related liver dysfunction *via* their antioxidant, antiviral, anti-inflammatory, and positive immunomodulatory effects. This review mainly focuses on the possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy to provide a reference for the nutritionists while making evidence-based nutritional decisions during the COVID-19 pandemic.

Key Words: COVID-19; SARS-CoV-2; Liver dysfunction; Nutritional status assessment; Nutrition therapy

Core Tip: Patients with coronavirus disease 2019 (COVID-19) usually have respiratory symptoms, but liver dysfunction is not an uncommon presentation. The degree of liver dysfunction is associated with COVID-19 severity and prognosis. Nutrition has played a critical therapeutic and prognostic role in the management of patients with COVID-19-related liver dysfunction. This review mainly focuses on the possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy in patients with COVID-19 to provide a reference for the nutritionists while making evidence-based nutritional decisions in the era of COVID-19.

INTRODUCTION

Since December 2019, novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been a major global health-related issue and has caused an unprecedented pandemic[1,2]. As of January 2, 2022, a total of 288867634 confirmed cases of COVID-19 and 5437636 deaths have been reported worldwide[3]. The liver is one of the main organs involved in nutrient metabolism, including protein synthesis, glycogen storage, and detoxification[4]. Studies have shown that angiotensin-converting enzyme 2 (ACE2), a functional receptor that allows the entry of SARS-CoV-2 into host cells, is expressed in cholangiocytes (59.7% of cells) and hepatocytes (2.6% of cells), indicating that COVID-19-related liver dysfunction may occur through direct cytotoxicity due to continuous viral replication within liver cells[5-8]. Additionally, COVID-19-related liver dysfunction was observed in approximately 20%-30% of the infected patients, especially in critically ill patients, and it was associated with poor outcomes[9-12]. Therefore, COVID-19-related liver dysfunction should not be ignored.

Meanwhile, almost all patients with liver disease, especially those at an advanced stage, have signs of malnutrition, including mineral and vitamin deficiency[13]. Micronutrient deficiencies may lead to impaired immune responses, including improper cytokine secretion, secretory antibody alterations, and antibody affinity, which increase the susceptibility to SARS-CoV-2 infection[14]. The malnutritional status of the host can also be a virulence factor for SARS-CoV-2 infection[15]. In addition, the nutritional status of COVID-19 patients with liver dysfunction is significantly related to the disease severity. An evaluation of the patient's nutritional status should not be ignored owing to the implications of nutritional status on the susceptibility, course, severity, and responsiveness to therapies[16,17]. Tailored nutritional therapy prescribed after evaluating the nutritional status has also been an integral part of the comprehensive treatment for patients with COVID-19. This paper mainly focuses on a possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy in patients with COVID-19.

COVID-19 AND LIVER DYSFUNCTION

Liver dysfunction has been reported in a significant proportion of COVID-19 patients, especially in those with a severe illness[5]. Additionally, 2%-11% of patients with COVID-19 suffer from chronic liver disease. The prevalence of liver dysfunction in COVID-19 ranged from 3.75% to 59.04%; most studies reported a prevalence of 20%-30%[9-12]. A cross-sectional study reported that the prevalence of liver dysfunction in patients with COVID-19 was 59.04%; of the 62 patients, 44 (70.9%) were male and 18 (29.03%) were female. The average hospital stay of patients with liver dysfunction was 15 d (range, 10-16 d) compared with 10 d (range, 7-11 d) for patients with normal liver function[12]. In general, patients with COVID-19 who develop liver dysfunction are mostly male, elderly, and obese[5]. Another retrospective cohort study reported that of the 2273 COVID-19 patients at three hospitals in the NewYork-Presbyterian network, 45% suffered from mild liver injury, 21% from moderate liver injury, and 6.4% from severe liver injury. Patients with severe liver dysfunction had elevated levels of inflammatory markers, including ferritin and interleukin-6. They also suffered a worse clinical course, including higher rates of intensive care unit (ICU) admission (69%), intubation (65%), renal replacement therapy (33%), and mortality (42%)[10]. Several studies reported that the presence of liver dysfunction was closely related to higher admission, as well as higher ICU admission and/or death. The measurement of liver biochemical indexes might help the clinicians to evaluate the severity and prognosis of patients with COVID-19[18-23]. The mechanisms of COVID-19-related liver dysfunction may include direct viral cytopathic impairment, secondary liver injury resulting from a systemic inflam-

matory response or hypoxia-reperfusion, stress-induced liver injury, drug-induced liver damage, and, finally, exacerbation of the pre-existing liver diseases (Figure 1)[5,24-27]. Although COVID-19-related liver injuries are often transient and reversible, physicians, dietitians, and nutritionists need to take notice of the pre-existing liver damage, monitor liver function, improve supportive treatment, and prevent the occurrence of drug-induced liver injury[28].

NUTRITIONAL AND METABOLIC CHARACTERISTICS OF PATIENTS WITH COVID-19

The main manifestation of patients with COVID-19 is high fever (range, 37.5-39.0 °C), which induces a catabolic state, including impaired glucose utilization, and increased energy utilization and protein breakdown. The metabolic effect of the temperature increase is said to be 10%-13% for every 1 °C increase, which should be considered in the nutritional recommendations[29]. Additionally, great influence on appetite and consciousness, and direct gastrointestinal damage may lead to nausea, vomiting, diarrhea, and feeding intolerance, which may adversely affect the nutrient intake and nutritional status[29]. Rouget *et al*[30] reported a high prevalence of malnutrition (37.5%) with 26% severe malnutrition according to the Global Leadership Initiative on Malnutrition (GLIM) criteria in a general cohort of patients with COVID-19. Bedock *et al*[31] reported that the overall incidence of malnutrition in COVID-19 patients was 42.1% (moderate: 23.7%; severe: 18.4%), while the incidence of malnutrition in patients admitted to the ICU reached 66.7% using the GLIM criteria. They found that lower albumin levels were related to a higher risk of admission to the ICU, and this association was independent of age and C-reactive protein levels. Li *et al*[32] found a high incidence (52.7%) of malnutrition according to the Mini Nutritional Assessment (MNA) in 182 elderly patients with COVID-19. Additionally, further regression analysis indicated that diabetes, low calf circumference, and low albumin level were independent risk factors for malnutrition. Malnutrition can impair the hepatic metabolic functions, and malnutrition alone can result in severe fatty liver[33].

NUTRITIONAL STATUS ASSESSMENT

According to the expert statements and practical guidance of the European Society for Clinical Nutrition and Metabolism for the nutritional management of individuals with SARS-CoV-2 infection, COVID-19 patients at risk of poor outcomes and high mortality, namely, the elderly and individuals with multiple comorbidities, should be assessed for malnutrition using the Malnutrition Universal Screening Tool (MUST) criteria; for hospitalized patients, the Nutrition Risk Screening 2002 (NRS-2002) criteria should be used[34]. Identification of the risk and presence of malnutrition should be conducted early in the overall assessment of all patients with COVID-19 using criteria such as MUST or NRS-2002. Ganatra *et al* [35] investigated and analyzed the nutritional risk and dietary intake of patients with COVID-19 and provided data supporting nutritional intervention using the NRS-2002 criteria. The Subjective Global Assessment criteria, the MNA criteria for geriatric patients, and the Nutrition Risk in Critically ill (NUTRIC) criteria for ICU patients have been used to further assess patients with COVID-19 and are accepted in clinical practice[36,37]. Zhang *et al*[38] reported that the modified NUTRIC score could be applied to nutritional risk evaluation and prognosis indication in critically ill patients with COVID-19 [39]. Recently, the GLIM criteria for malnutrition diagnosis endorsed by clinical nutrition societies worldwide have been used to assess the nutritional status of patients with COVID-19[30,31].

NUTRITION THERAPY FOR COVID-19-RELATED LIVER DYSFUNCTION

Currently, the fight against the COVID-19 epidemic is entering a decisive stage[38]. Evidence-based and logical nutrition interventions can effectively improve the nutritional status and enhance the immunity, and they are essential for preventing and managing viral infections[40]. Patients with mild clinical manifestations or recovered patients who have returned home should rest in bed, carefully choose foods and recipes, maintain an adequate supply of energy and nutrients (including drinking water), improve their immune status, and speed up their recovery process. Severe, critically ill patients with COVID-19 often have loss of appetite and insufficient diet, which worsens their already weak immune system. For these patients, subsequent nutritional support should be adopted, and specific nutritional treatment plans should be formulated according to the general condition of the patient's body, fluid intake, liver and kidney functions, and glucose and lipid metabolism[41]. Ten expert recommendations for medical nutritional therapy for patients with COVID-19 have been proposed by the Chinese Society for Parenteral and Enteral Nutrition to further promote patient recovery, improve their treatment effects, and reduce the mortality rate[42]. The main nutritional therapy recommendation is a five-step method, including diet and nutrition education, oral nutritional supplements, tube feeding, supplemental parenteral nutrition, and total parenteral nutrition[42].
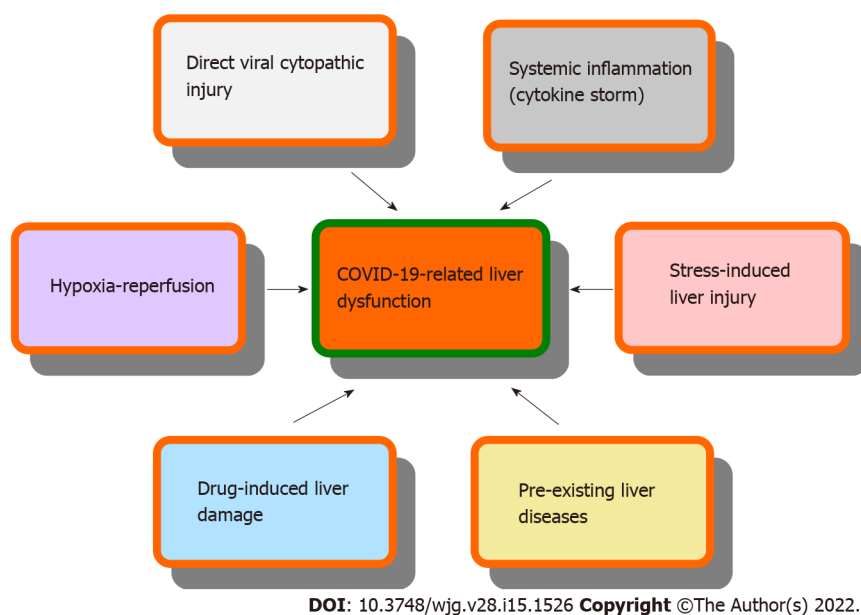
Figure 1 Mechanisms of coronavirus disease 2019-related liver dysfunction. COVID-19: Coronavirus disease 2019.

Numerous studies have found that nutrition therapy measures for patients with COVID-19 mainly include natural dietary supplements, vitamins, minerals, trace elements, and probiotics. A schematic summarizing the nutritional interventions for COVID-19-related liver dysfunction and their possible mechanisms is shown in Figure 2.

Natural dietary supplements

Rizzo *et al*[43] reviewed the properties of some vegetal products and their derivatives, including Lupin, salvia, garlic, and extra-virgin olive oil (EVOO), and they found that intake of these products or their extracts might prevent SARS-CoV-2 infection or improve the patients' nutritional status. Lupin, salvia, garlic, and EVOO have anti-oxidant, anti-inflammatory, or antiviral properties and can recover the expression of ACE2 on the cell membrane, otherwise suppressed by SARS-CoV-2 binding and entry into the cytoplasm. Sikander *et al*[6] provided information and summarized the effects of natural bioactive antiviral, immunomodulatory, and hepatoprotective nutraceuticals (*Silybum marianum*, *Solanum nigrum*, *Cichorium intybus*, *Allium sativum*, *Glycyrrhiza glabra*, *Phyllanthus amarus*, *Withania somnifera*, *Curcuma longa*, and other hepatoprotective agents) that might be explored in managing COVID-19-induced liver dysfunction. Additionally, omega-3 long-chain polyunsaturated fatty acids (omega-3 LC-PUFAs) might also have effects on different stages of viral infection, including virus entry and replication, and help improve the inflammatory balance. An optimized omega-3 PUFA status, considering both the omega-3 precursor alpha-linolenic acid and long-chain derivatives, such as eicosapentaenoic acid and docosahexaenoic acid, might be helpful in preventing infectious diseases, including COVID-19[44].

Vitamins

Accumulating data have demonstrated that vitamin deficiency could be a risk factor for SARS-CoV-2 infection and it affects the COVID-19 susceptibility and prognosis[45]. For instance, vitamin A deficiency increases the severity of the disease, and appropriately timed intake during recovery reduces the death risk and speeds up the recovery. Studying interactions of vitamin A metabolism with SARS-CoV-2 infection may thus provide improved COVID-19 treatment[46]. Vitamin D deficiency may decrease the ability of the immune system to defend against COVID-19 and cause progression to severe disease[47]. Vitamin K deficiency may be a potentially modifiable risk factor for severe COVID-19; the mechanism is pneumonia-induced extrahepatic vitamin K consumption, resulting in accelerated elastic fiber damage and thrombosis[48]. Vitamins A, B, C, D, and E have been shown to be potentially beneficial in fighting against COVID-19 by exerting antioxidant and immunomodulatory effects, increasing natural barriers, and causing local paracrine signaling[49]. Additionally, vitamins can serve as epigenetic modifiers to enhance the immunity and reduce the inflammatory response in patients with COVID-19 and noncommunicable diseases. Combined vitamin therapy can improve the health in a more personalized manner or help in the prevention of infectious diseases in patients at risk for COVID-19[50]. Molecular simulations also suggest that vitamins, steroids, and retinoids may serve as ligands in the free fatty acid pocket of the SARS-CoV-2 spike protein and may thus provide a promising strategy for prophylaxis or therapeutics[51]. Supplements with vitamins A, B, C, D, E, and K may represent a cheap and safe approach and can be used as adjuvant therapy together with antiviral medicines in managing COVID-19[45,52]. However, caution must be exercised when recommending vitamin supple-
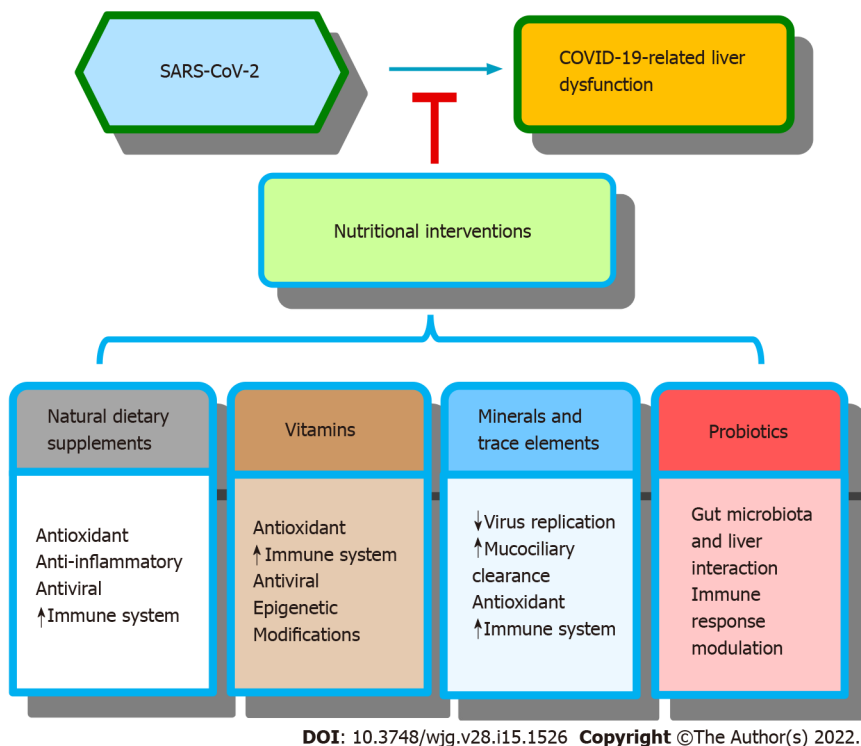
Figure 2 Scheme showing the effects of nutritional interventions against coronavirus disease 2019-related liver dysfunction and their possible mechanisms. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; COVID-19: Coronavirus disease 2019.

mentation during the COVID-19 pandemic because the effects of hypervitaminosis can be serious, especially of fat-soluble vitamins A, D, and E[49]. More robust data from randomized controlled trials are needed in the near future.

Minerals and trace elements

Zinc (Zn) supplementation may inhibit SARS-CoV-2 virus replication, increase mucociliary clearance, and stimulate and activate the natural antiviral response of the immune system in patients with COVID-19[52]. Derwand *et al*[53] have also reported that the application of the combination therapy of Zn, low-dose hydroxychloroquine, and azithromycin to COVID-19 outpatients as early as possible after symptom onset resulted in significantly fewer hospitalizations and mortality rates.

Selenium (Se), one of the essential trace elements in the human body, has antioxidant and immunity-boosting effects that may induce a strong antiviral effect[54]. Recently, it was reported that the Se status was positively correlated with the survival rate of COVID-19 patients according to an exploratory study on the Se status in healthy individuals and patients with COVID-19 in the southern part of India[55]. Se has long been considered to help fight different viruses, such as herpes simplex virus type 1, influenza virus, Coxsackie virus, hepatitis C virus, and human immunodeficiency virus; the potential application of nano-Se may play an important role in combating COVID-19 in the future[54]. Nutrition interventions that ensure an adequate status of Zn, Se, and vitamin D could protect against infection with the novel coronavirus SARS-CoV-2 and retard the course of COVID-19. Meanwhile, the initiation of adequate supplementation of Se in high-risk population immediately after the time of suspected SARS-CoV-2 infection is recommended[56].

Magnesium (Mg) is important for the activation of vitamin D and plays a protective role against oxidative stress. Mg deficiency increases the endothelial cell susceptibility to oxidative stress, induces endothelial dysfunction, decreases fibrinolysis, and increases coagulation. Mg-deficiency in animals and humans may lead to suppressed immune responses. However, upon supplementation with Mg, a partial or nearly full reversal of immunodeficiency occurs. Since Mg and vitamin D are important for immune function and cellular resilience, deficiency of either of the two micronutrients may contribute to cytokine storms in COVID-19 infection[57]. Furthermore, a low Mg status may induce the transition from mild to critical clinical manifestations of COVID-19[58]. Additionally, a recent review summarized the effect of Mg supplementation on various types of disorders and diseases, providing a reference supporting the possibility of Mg supplementation for supportive therapy of COVID-19 patients[59]. Additional epidemiological, basic, and clinical research on the potential role of Mg deficiency in COVID-19 is needed.

Copper (Cu) is an important micronutrient for both pathogens and hosts during viral infection. It has the capability of contact killing of several viruses, including SARS-CoV-2[60]. Enrichment of plasma Cu

levels was hypothesized to boost both innate and adaptive immunity; Cu may have preventive and therapeutic effects against COVID-19[60]. A better understanding of Cu signaling, safety, assessment and interpretation methods, administration route, and dosage could open up new perspectives regarding the administration of therapeutic Cu to critically ill patients with COVID-19. Andreou *et al*[61] found that the combined use of Cu, colchicine, N-acetylcysteine, and nitric oxide (NO) with candidate antiviral agents, such as remdesivir or EIDD-2801, might be a potential treatment scheme for COVID-19. Physicians should consider Cu insufficiency in critically ill patients with COVID-19 and pay attention to Cu toxicity and estimate the adverse responses according to the Cu dose, and severity of Cu limitation, as well as the duration of Cu imbalance[62].

Probiotics
Beneficial live microbes in humans and animals are known as probiotics, and the chemical compounds that increase the probiotic growth rate are termed prebiotics[63]. SARS-CoV-2 infection is closely related to immune dysfunction and gut microbiota alterations. Delineating the mechanisms of probiotics, prebiotics, and a diet that promotes immunity and protects against SARS-CoV-2 presents possibilities of identifying microbial therapies to prevent and treat COVID-19[64]. Probiotics can exert beneficial effects by manipulating the gut microbiome, suppressing the gut opportunistic pathogens, decreasing the translocation of opportunistic organisms in the gut, activating the mucosal immunity, and modulating the innate and adaptive immune responses. Probiotics may be used as potential candidates to treat moderate and severe COVID-19 patients due to their benefits, including safety, ease of administration, high availability, and cost-effectiveness[65]. Emerging evidence has shown the role of gut microbiota in liver diseases through immune system cross-talk[66]. There is a lack of evidence that probiotics can directly inhibit SARS-CoV-2 infection, and probiotic therapy in COVID-19-related liver dysfunction is also not very effective. However, probiotics may be potentially helpful in the treatment of patients with severe COVID-19 and liver dysfunction[67].

CONCLUSION

Patients with COVID-19 usually have respiratory symptoms, but liver dysfunction is not an uncommon presentation and can lead to a delay in diagnosis and management[68]. Nutrition and immune statuses are two critical aspects of the successful fight against COVID-19[55]. Prevention, diagnosis, and treatment of malnutrition should be routinely recommended in the management of patients with COVID-19, especially in those with liver dysfunction[34,69]. Nutritional therapy is a basic treatment and one of the core contents of comprehensive treatment measures for patients with COVID-19. Evidence-based effective nutritional therapy should be based on reasonable and indexed nutritional evaluation [70].

Studies have shown that nutrition therapy measures, including natural dietary supplements, vitamins, minerals, trace elements, and probiotics, might have potential hepatoprotective effects against COVID-19-related liver dysfunction *via* their antioxidant, antiviral, anti-inflammatory, and positive immunomodulatory effects. Combination therapy strategies and personalized nutritional and behavioral approaches can be developed in the COVID-19 era[52,71]. Additionally, the risk of excessive intake of some nutrients due to the popularity of dietary supplements exists, and dietitians' use of foods with protective effects against diseases has increased during the pandemic. Hence, consumers, patients, and nutritionists should be educated on the rational use of dietary supplements and health-protecting behaviors that can protect against COVID-19 for acute treatment, recovery, and prevention of chronic condition[72,73]. Moreover, additional tools and training are needed to optimize remote nutritional consultations, except for telemedicine, which have good prospects for dietary consultation[74]. Currently, many patients with COVID-19 have liver dysfunction, but nutritional studies related to this topic are not adequate. As nutritionists, it is our responsibility and obligation to facilitate further research in this area.

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REFERENCES

1　**Abdulrab S**, Al-Maweri S, Halboub E. Ursodeoxycholic acid as a candidate therapeutic to alleviate and/or prevent COVID-19-associated cytokine storm. *Med Hypotheses* 2020; **143**: 109897 [PMID: 32505909 DOI: 10.1016/j.mehy.2020.109897]

2　**Mahase E**. Covid-19: WHO declares pandemic because of "alarming levels" of spread, severity, and inaction. *BMJ* 2020; **368**: m1036 [PMID: 32165426 DOI: 10.1136/bmj.m1036]

3　**World Health Organization**.　Weekly epidemiological update on COVID-19 - 6 January 2022. [cited 6 January 2022]. Available from: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---6-january-2022

4　**Yasutake K**, Kohjima M, Nakashima M, Kotoh K, Nakamuta M, Enjoji M. Nutrition therapy for liver diseases based on the status of nutritional intake. *Gastroenterol Res Pract* 2012; **2012**: 859697 [PMID: 23197979 DOI: 10.1155/2012/859697]

5　**Cichoż-Lach H**, Michalak A. Liver injury in the era of COVID-19. *World J Gastroenterol* 2021; **27**: 377-390 [PMID: 33584070 DOI: 10.3748/wjg.v27.i5.377]

6　**Sikander M**, Malik S, Rodriguez A, Yallapu MM, Narula AS, Satapathy SK, Dhevan V, Chauhan SC, Jaggi M. Role of Nutraceuticals in COVID-19 Mediated Liver Dysfunction. *Molecules* 2020; **25** [PMID: 33322162 DOI: 10.3390/molecules25245905]

7　**Loganathan S**, Kuppusamy M, Wankhar W, Gurugubelli KR, Mahadevappa VH, Lepcha L, Choudhary AK. Angiotensin-converting enzyme 2 (ACE2): COVID 19 gate way to multiple organ failure syndromes. *Respir Physiol Neurobiol* 2021; **283**: 103548 [PMID: 32956843 DOI: 10.1016/j.resp.2020.103548]

8　**Hoffmann M**, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**: 271-280.e8 [PMID: 32142651 DOI: 10.1016/j.cell.2020.02.052]

9　**Merola E**, Pravadelli C, de Pretis G. Prevalence of liver injury in patients with coronavirus disease 2019 (COVID-19): a systematic review and meta-analysis. *Acta Gastroenterol Belg* 2020; **83**: 454-460 [PMID: 33094594]

10　**Phipps MM**, Barraza LH, LaSota ED, Sobieszczyk ME, Pereira MR, Zheng EX, Fox AN, Zucker J, Verna EC. Acute Liver Injury in COVID-19: Prevalence and Association with Clinical Outcomes in a Large U.S. Cohort. *Hepatology* 2020; **72**: 807-817 [PMID: 32473607 DOI: 10.1002/hep.31404]

11　**Jothimani D**, Venugopal R, Abedin MF, Kaliamoorthy I, Rela M. COVID-19 and the liver. *J Hepatol* 2020; **73**: 1231-1240 [PMID: 32553666 DOI: 10.1016/j.jhep.2020.06.006]

12　**Kaushik A**, Wani SN, Baba MA, Agarwal AK. Prevalence of Abnormal Liver Function Tests in COVID-19 Patients at a Tertiary Care Centre. *J Assoc Physicians India* 2020; **68**: 73-75 [PMID: 32738845]

13　**Hanje AJ**, Fortune B, Song M, Hill D, McClain C. The use of selected nutrition supplements and complementary and alternative medicine in liver disease. *Nutr Clin Pract* 2006; **21**: 255-272 [PMID: 16772543 DOI: 10.1177/0115426506021003255]

14　**Muthuvattur Pallath M**, Ahirwar AK, Chandra Tripathi S, Asia P, Sakarde A, Gopal N. COVID-19 and nutritional deficiency: a review of existing knowledge. *Horm Mol Biol Clin Investig* 2021; **42**: 77-85 [PMID: 33544528 DOI: 10.1515/hmbci-2020-0074]

15　**Briguglio M**, Pregliasco FE, Lombardi G, Perazzo P, Banfi G. The Malnutritional Status of the Host as a Virulence Factor for New Coronavirus SARS-CoV-2. *Front Med (Lausanne)* 2020; **7**: 146 [PMID: 32391367 DOI: 10.3389/fmed.2020.00146]

16　**Cai CJ**. Nutritional therapy in patients with liver dysfunction.　*Chin J Pract Surg* 2019; **39**: 79-84 [DOI: 10.19538/j.cjps.issn1005-2208.2019.01.14]

17　**Fedele D**, De Francesco A, Riso S, Collo A. Obesity, malnutrition, and trace element deficiency in the coronavirus disease (COVID-19) pandemic: An overview. *Nutrition* 2021; **81**: 111016 [PMID: 33059127 DOI: 10.1016/j.nut.2020.111016]

18　**Wu Y**, Li H, Guo X, Yoshida EM, Mendez-Sanchez N, Levi Sandri GB, Teschke R, Romeiro FG, Shukla A, Qi X.

Incidence, risk factors, and prognosis of abnormal liver biochemical tests in COVID-19 patients: a systematic review and meta-analysis. *Hepatol Int* 2020; **14**: 621-637 [PMID: 32710250 DOI: 10.1007/s12072-020-10074-6]

19   **Ye L**, Chen B, Wang Y, Yang Y, Zeng J, Deng G, Deng Y, Zeng F. Prognostic value of liver biochemical parameters for COVID-19 mortality. *Ann Hepatol* 2021; **21**: 100279 [PMID: 33157267 DOI: 10.1016/j.aohep.2020.10.007]

20   **Lin L**, Jiang X, Zhang Z, Huang S, Fang Z, Gu Z, Gao L, Shi H, Mai L, Liu Y, Lin X, Lai R, Yan Z, Li X, Shan H. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. *Gut* 2020; **69**: 997-1001 [PMID: 32241899 DOI: 10.1136/gutjnl-2020-321013]

21   **Zhou F**, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 2020; **395**: 1054-1062 [PMID: 32171076 DOI: 10.1016/S0140-6736(20)30566-3]

22   **Hajifathalian K**, Krisko T, Mehta A, Kumar S, Schwartz R, Fortune B, Sharaiha RZ; WCM-GI research group□. Gastrointestinal and Hepatic Manifestations of 2019 Novel Coronavirus Disease in a Large Cohort of Infected Patients From New York: Clinical Implications. *Gastroenterology* 2020; **159**: 1137-1140.e2 [PMID: 32389667 DOI: 10.1053/j.gastro.2020.05.010]

23   **Chen T**, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, Ding C, Zhang X, Huang J, Han M, Li S, Luo X, Zhao J, Ning Q. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ* 2020; **368**: m1091 [PMID: 32217556 DOI: 10.1136/bmj.m1091]

24   **Huang C**, Li Q, Xu W, Chen L. Molecular and cellular mechanisms of liver dysfunction in COVID-19. *Discov Med* 2020; **30**: 107-112 [PMID: 33382966]

25   **Lizardo-Thiebaud MJ**, Cervantes-Alvarez E, Limon-de la Rosa N, Tejeda-Dominguez F, Palacios-Jimenez M, Méndez-Guerrero O, Delaye-Martinez M, Rodriguez-Alvarez F, Romero-Morales B, Liu WH, Huang CA, Kershenobich D, Navarro-Alvarez N. Direct or Collateral Liver Damage in SARS-CoV-2-Infected Patients. *Semin Liver Dis* 2020; **40**: 321-330 [PMID: 32886936 DOI: 10.1055/s-0040-1715108]

26   **Li J**, Fan JG. Characteristics and Mechanism of Liver Injury in 2019 Coronavirus Disease. *J Clin Transl Hepatol* 2020; **8**: 13-17 [PMID: 32274341 DOI: 10.14218/JCTH.2020.00019]

27   **Zhong P**, Xu J, Yang D, Shen Y, Wang L, Feng Y, Du C, Song Y, Wu C, Hu X, Sun Y. COVID-19-associated gastrointestinal and liver injury: clinical features and potential mechanisms. *Signal Transduct Target Ther* 2020; **5**: 256 [PMID: 33139693 DOI: 10.1038/s41392-020-00373-7]

28   **Yang RX**, Zheng RD, Fan JG. Etiology and management of liver injury in patients with COVID-19. *World J Gastroenterol* 2020; **26**: 4753-4762 [PMID: 32921955 DOI: 10.3748/wjg.v26.i32.4753]

29   **Chapple LS**, Fetterplace K, Asrani V, Burrell A, Cheng AC, Collins P, Doola R, Ferrie S, Marshall AP, Ridley EJ. Nutrition management for critically and acutely unwell hospitalised patients with coronavirus disease 2019 (COVID-19) in Australia and New Zealand. *Aust Crit Care* 2020; **33**: 399-406 [PMID: 32682671 DOI: 10.1016/j.aucc.2020.06.002]

30   **Rouget A**, Vardon-Bounes F, Lorber P, Vavasseur A, Marion O, Marcheix B, Lairez O, Balardy L, Fourcade O, Conil JM, Minville V. Prevalence of malnutrition in coronavirus disease 19: the NUTRICOV study. *Br J Nutr* 2021; **126**: 1296-1303 [PMID: 33342449 DOI: 10.1017/S0007114520005127]

31   **Bedock D**, Bel Lassen P, Mathian A, Moreau P, Couffignal J, Ciangura C, Poitou-Bernert C, Jeannin AC, Mosbah H, Fadlallah J, Amoura Z, Oppert JM, Faucher P. Prevalence and severity of malnutrition in hospitalized COVID-19 patients. *Clin Nutr ESPEN* 2020; **40**: 214-219 [PMID: 33183539 DOI: 10.1016/j.clnesp.2020.09.018]

32   **Li T**, Zhang Y, Gong C, Wang J, Liu B, Shi L, Duan J. Prevalence of malnutrition and analysis of related factors in elderly patients with COVID-19 in Wuhan, China. *Eur J Clin Nutr* 2020; **74**: 871-875 [PMID: 32322046 DOI: 10.1038/s41430-020-0642-3]

33   **Bischoff SC**, Bernal W, Dasarathy S, Merli M, Plank LD, Schütz T, Plauth M. ESPEN practical guideline: Clinical nutrition in liver disease. *Clin Nutr* 2020; **39**: 3533-3562 [PMID: 33213977 DOI: 10.1016/j.clnu.2020.09.001]

34   **Barazzoni R**, Bischoff SC, Breda J, Wickramasinghe K, Krznaric Z, Nitzan D, Pirlich M, Singer P; endorsed by the ESPEN Council. ESPEN expert statements and practical guidance for nutritional management of individuals with SARS-CoV-2 infection. *Clin Nutr* 2020; **39**: 1631-1638 [PMID: 32305181 DOI: 10.1016/j.clnu.2020.03.022]

35   **Ganatra S**, Hammond SP, Nohria A. The Novel Coronavirus Disease (COVID-19) Threat for Patients With Cardiovascular Disease and Cancer. *JACC CardioOncol* 2020; **2**: 350-355 [PMID: 32292919 DOI: 10.1016/j.jaccao.2020.03.001]

36   **Martins PM**, Gomes TLN, Franco EP, Vieira LL, Pimentel GD. High neutrophil-to-lymphocyte ratio at intensive care unit admission is associated with nutrition risk in patients with COVID-19. *JPEN J Parenter Enteral Nutr* 2021 [PMID: 34961953 DOI: 10.1002/jpen.2318]

37   **Ali AM**, Kunugi H. Approaches to Nutritional Screening in Patients with Coronavirus Disease 2019 (COVID-19). *Int J Environ Res Public Health* 2021; **18** [PMID: 33803339 DOI: 10.3390/ijerph18052772]

38   **Zhang P**, He Z, Yu G, Peng D, Feng Y, Ling J, Wang Y, Li S, Bian Y. The modified NUTRIC score can be used for nutritional risk assessment as well as prognosis prediction in critically ill COVID-19 patients. *Clin Nutr* 2021; **40**: 534-541 [PMID: 32527576 DOI: 10.1016/j.clnu.2020.05.051]

39   **Nutrition Management in Critically Ill Project Team**, Chinese Nutrition Society for Clinical Nutrition. Recommendations for nutrition therapy in critically ill COVID-19 patients. *Chin J Clin Med* 2020; **27**: 167-174 [DOI: 10.12025/j.issn.1008-6358.2020.]

40   **Jayawardena R**, Sooriyaarachchi P, Chourdakis M, Jeewandara C, Ranasinghe P. Enhancing immunity in viral infections, with special emphasis on COVID-19: A review. *Diabetes Metab Syndr* 2020; **14**: 367-382 [PMID: 32334392 DOI: 10.1016/j.dsx.2020.04.015]

41   **Law S**, Leung AW, Xu C. Severe acute respiratory syndrome (SARS) and coronavirus disease-2019 (COVID-19): From causes to preventions in Hong Kong. *Int J Infect Dis* 2020; **94**: 156-163 [PMID: 32251790 DOI: 10.1016/j.ijid.2020.03.059]

42   **Chinese Society for Parenteral and Enteral Nutrition**. Expert advice of medical nutritional treatment for novel coronavirus-caused pneumonia patients. *Chin Arch Gen Surg* 2020; **14**: 1 [DOI:

10.3877/cma.j.issn.1674-0793.2020.01.001]

43   **Rizzo A**, Sciorsci RL, Magrone T, Jirillo E. Exploitation of Some Natural Products for the Prevention and/or Nutritional Treatment of SARS-CoV2 Infection. *Endocr Metab Immune Disord Drug Targets* 2021; **21**: 1171-1182 [PMID: 32875990 DOI: 10.2174/1871530320999200831231029]

44   **Weill P**, Plissonneau C, Legrand P, Rioux V, Thibault R. May omega-3 fatty acid dietary supplementation help reduce severe complications in Covid-19 patients? *Biochimie* 2020; **179**: 275-280 [PMID: 32920170 DOI: 10.1016/j.biochi.2020.09.003]

45   **Allegra A**, Tonacci A, Pioggia G, Musolino C, Gangemi S. Vitamin deficiency as risk factor for SARS-CoV-2 infection: correlation with susceptibility and prognosis. *Eur Rev Med Pharmacol Sci* 2020; **24**: 9721-9738 [PMID: 33015818 DOI: 10.26355/eurrev_202009_23064]

46   **Stephensen CB**, Lietz G. Vitamin A in resistance to and recovery from infection: relevance to SARS-CoV2. *Br J Nutr* 2021; **126**: 1663-1672 [PMID: 33468263 DOI: 10.1017/S0007114521000246]

47   **Im JH**, Je YS, Baek J, Chung MH, Kwon HY, Lee JS. Nutritional status of patients with COVID-19. *Int J Infect Dis* 2020; **100**: 390-393 [PMID: 32795605 DOI: 10.1016/j.ijid.2020.08.018]

48   **Dofferhoff ASM**, Piscaer I, Schurgers LJ, Visser MPJ, van den Ouweland JMW, de Jong PA, Gosens R, Hackeng TM, van Daal H, Lux P, Maassen C, Karssemeijer EGA, Vermeer C, Wouters EFM, Kistemaker LEM, Walk J, Janssen R. Reduced Vitamin K Status as a Potentially Modifiable Risk Factor of Severe Coronavirus Disease 2019. *Clin Infect Dis* 2021; **73**: e4039-e4046 [PMID: 32852539 DOI: 10.1093/cid/ciaa1258]

49   **Jovic TH**, Ali SR, Ibrahim N, Jessop ZM, Tarassoli SP, Dobbs TD, Holford P, Thornton CA, Whitaker IS. Could Vitamins Help in the Fight Against COVID-19? *Nutrients* 2020; **12** [PMID: 32842513 DOI: 10.3390/nu12092550]

50   **Singh V**. Can Vitamins, as Epigenetic Modifiers, Enhance Immunity in COVID-19 Patients with Non-communicable Disease? *Curr Nutr Rep* 2020; **9**: 202-209 [PMID: 32661859 DOI: 10.1007/s13668-020-00330-4]

51   **Shoemark DK**, Colenso CK, Toelzer C, Gupta K, Sessions RB, Davidson AD, Berger I, Schaffitzel C, Spencer J, Mulholland AJ. Molecular Simulations suggest Vitamins, Retinoids and Steroids as Ligands of the Free Fatty Acid Pocket of the SARS-CoV-2 Spike Protein*. *Angew Chem Int Ed Engl* 2021; **60**: 7098-7110 [PMID: 33469977 DOI: 10.1002/anie.202015639]

52   **Islam MT**, Quispe C, Martorell M, Docea AO, Salehi B, Calina D, Reiner Ž, Sharifi-Rad J. Dietary supplements, vitamins and minerals as potential interventions against viruses: Perspectives for COVID-19. *Int J Vitam Nutr Res* 2022; **92**: 49-66 [PMID: 33435749 DOI: 10.1024/0300-9831/a000694]

53   **Derwand R**, Scholz M, Zelenko V. COVID-19 outpatients: early risk-stratified treatment with zinc plus low-dose hydroxychloroquine and azithromycin: a retrospective case series study. *Int J Antimicrob Agents* 2020; **56**: 106214 [PMID: 33122096 DOI: 10.1016/j.ijantimicag.2020.106214]

54   **He L**, Zhao J, Wang L, Liu Q, Fan Y, Li B, Yu YL, Chen C, Li YF. Using nano-selenium to combat Coronavirus Disease 2019 (COVID-19)? *Nano Today* 2021; **36**: 101037 [PMID: 33250930 DOI: 10.1016/j.nantod.2020.101037]

55   **Majeed M**, Nagabhushanam K, Gowda S, Mundkur L. An exploratory study of selenium status in healthy individuals and in patients with COVID-19 in a south Indian population: The case for adequate selenium status. *Nutrition* 2021; **82**: 111053 [PMID: 33321395 DOI: 10.1016/j.nut.2020.111053]

56   **Alexander J**, Tinkov A, Strand TA, Alehagen U, Skalny A, Aaseth J. Early Nutritional Interventions with Zinc, Selenium and Vitamin D for Raising Anti-Viral Resistance Against Progressive COVID-19. *Nutrients* 2020; **12** [PMID: 32784601 DOI: 10.3390/nu12082358]

57   **DiNicolantonio JJ**, O'Keefe JH. Magnesium and Vitamin D Deficiency as a Potential Cause of Immune Dysfunction, Cytokine Storm and Disseminated Intravascular Coagulation in covid-19 patients. *Mo Med* 2021; **118**: 68-73 [PMID: 33551489]

58   **Iotti S**, Wolf F, Mazur A, Maier JA. The COVID-19 pandemic: is there a role for magnesium? *Magnes Res* 2020; **33**: 21-27 [PMID: 32554340 DOI: 10.1684/mrh.2020.0465]

59   **Tang CF**, Ding H, Jiao RQ, Wu XX, Kong LD. Possibility of magnesium supplementation for supportive treatment in patients with COVID-19. *Eur J Pharmacol* 2020; **886**: 173546 [PMID: 32931782 DOI: 10.1016/j.ejphar.2020.173546]

60   **Raha S**, Mallick R, Basak S, Duttaroy AK. Is copper beneficial for COVID-19 patients? *Med Hypotheses* 2020; **142**: 109814 [PMID: 32388476 DOI: 10.1016/j.mehy.2020.109814]

61   **Andreou A**, Trantza S, Filippou D, Sipsas N, Tsiodras S. COVID-19: The Potential Role of Copper and N-acetylcysteine (NAC) in a Combination of Candidate Antiviral Treatments Against SARS-CoV-2. *In Vivo* 2020; **34**: 1567-1588 [PMID: 32503814 DOI: 10.21873/invivo.11946]

62   **Fooladi S**, Matin S, Mahmoodpoor A. Copper as a potential adjunct therapy for critically ill COVID-19 patients. *Clin Nutr ESPEN* 2020; **40**: 90-91 [PMID: 33183578 DOI: 10.1016/j.clnesp.2020.09.022]

63   **Khaled JMA**. Probiotics, prebiotics, and COVID-19 infection: A review article. *Saudi J Biol Sci* 2021; **28**: 865-869 [PMID: 33424377 DOI: 10.1016/j.sjbs.2020.11.025]

64   **Hu J**, Zhang L, Lin W, Tang W, Chan FKL, Ng SC. Review article: Probiotics, prebiotics and dietary approaches during COVID-19 pandemic. *Trends Food Sci Technol* 2021; **108**: 187-196 [PMID: 33519087 DOI: 10.1016/j.tifs.2020.12.009]

65   **Angurana SK**, Bansal A. Probiotics and Coronavirus disease 2019: think about the link. *Br J Nutr* 2021; **126**: 1564-1570 [PMID: 32921328 DOI: 10.1017/S000711452000361X]

66   **Scarpellini E**, Fagoonee S, Rinninella E, Rasetti C, Aquila I, Larussa T, Ricci P, Luzza F, Abenavoli L. Gut Microbiota and Liver Interaction through Immune System Cross-Talk: A Comprehensive Review at the Time of the SARS-CoV-2 Pandemic. *J Clin Med* 2020; **9** [PMID: 32756323 DOI: 10.3390/jcm9082488]

67   **Aguila EJT**, Lontok MADC, Aguila EJT. Letter: role of probiotics in the COVID-19 pandemic. *Aliment Pharmacol Ther* 2020; **52**: 931-932 [PMID: 32852829 DOI: 10.1111/apt.15898]

68   **Kamani L**. What gastroenterologists should know during COVID-19 Pandemic! *Pak J Med Sci* 2020; **36**: S124-S125 [PMID: 32582330 DOI: 10.12669/pjms.36.COVID19-S4.2651]

69   **Cervantes-Pérez E**, Cervantes-Guevara G, Martínez-Soto Holguín MC, Cervantes-Pérez LA, Cervantes-Pérez G,

Cervantes-Cardona GA, González-Ojeda A, Fuentes-Orozco C, Ramírez-Ochoa S. Medical Nutrition Therapy in Hospitalized Patients With SARS-CoV-2 (COVID-19) Infection in a Non-critical Care Setting: Knowledge in Progress. *Curr Nutr Rep* 2020; **9**: 309-315 [PMID: 33125628 DOI: 10.1007/s13668-020-00337-x]

70   **Yu KY**, Shi HP. [Explanation of expert recommendations on medical nutrition for patients with novel coronavirus pneumonia]. *Zhonghua Yi Xue Za Zhi* 2020; **100**: 724-728 [PMID: 32192285 DOI: 10.3760/cma.j.cn112137-20200205-00196]

71   **Formisano E**, Di Maio P, Ivaldi C, Sferrazzo E, Arieta L, Bongiovanni S, Panizzi L, Valentino E, Pasta A, Giudice M, Demontis S. Nutritional therapy for patients with coronavirus disease 2019 (COVID-19): Practical protocol from a single center highly affected by an outbreak of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. *Nutrition* 2021; **82**: 111048 [PMID: 33277149 DOI: 10.1016/j.nut.2020.111048]

72   **Hamulka J**, Jeruszka-Bielak M, Górnicka M, Drywień ME, Zielinska-Pukos MA. Dietary Supplements during COVID-19 Outbreak. Results of Google Trends Analysis Supported by PLifeCOVID-19 Online Studies. *Nutrients* 2020; **13** [PMID: 33375422 DOI: 10.3390/nu13010054]

73   **Kamarli Altun H**, Karacil Ermumcu MS, Seremet Kurklu N. Evaluation of dietary supplement, functional food and herbal medicine use by dietitians during the COVID-19 pandemic. *Public Health Nutr* 2021; **24**: 861-869 [PMID: 33357253 DOI: 10.1017/S1368980020005297]

74   **Kaufman-Shriqui V**, Sherf-Dagan S, Boaz M, Birk R. Virtual nutrition consultation: what can we learn from the COVID-19 pandemic? *Public Health Nutr* 2021; **24**: 1166-1173 [PMID: 33436134 DOI: 10.1017/S1368980021000148]

ORIGINAL ARTICLE

Basic Study

Establishing a rabbit model of perianal fistulizing Crohn's disease

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Abstract

BACKGROUND
Crohn's disease (CD) is a chronic nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear. Anal fistula is the main complication of CD and is a difficult problem to solve at present. The main limitation of developing new therapies is bound up with the short of preclinical security and effectiveness data. Therefore, an ideal animal model is needed to establish persistent anal fistula and an inflamed rectal mucosa.

AIM
To improve the induction method of colitis and establish a reliable and reproducible perianal fistulizing Crohn's disease animal model to evaluate new treatment strategies.

METHODS
Twenty male New Zealand rabbits underwent rectal enema with different doses of 2,4,6-trinitrobenzene sulfonic acid to induce proctitis. Group A was treated with an improved equal interval small dose increasing method. The dosage of group B was constant. Seven days later, the rabbits underwent surgical creation of a transsphincteric fistula. Then, three rabbits were randomly selected from each

group every 7 d to remove the seton from the fistula. The rabbits were examined by endoscopy every 7 days, and biopsy forceps were used to obtain tissue samples from the obvious colon lesions for histological analysis. The disease activity index (DAI), colonoscopy and histological scores were recorded. Perianal endoscopic ultrasonography (EUS) was used to evaluate the healing of fistulas.

RESULTS
Except for the DAI score, the colonoscopy and histological scores in group A were significantly higher than those in group B ($P < 0.05$). In the ideal model rabbit group, on the 7th day after the removal of the seton, all animals had persistent lumens on EUS imaging, showing continuous full-thickness high signals. Histological inspection of the fistula showed acute and chronic inflammation, fibrosis, epithelialization and peripheral proctitis of the adjoining rectum.

CONCLUSION
The improved method of CD colitis induction successfully established a rabbit perianal fistula CD preclinical model, which was confirmed by endoscopy and pathology.

Key Words: Crohn's disease; Perianal fistula; Model; Endoscopy; Histology

Core Tip: In this work, we improved the method of Crohn's disease (CD) colitis induction and successfully established a rabbit perianal fistula CD preclinical model, which was confirmed by endoscopy and pathology. The anatomy of this mid- to large-sized animal can simulate the human intestinal environment and tolerate examination and operation. This model may be used to assess perianal fistulizing CD treatments and their effectiveness.

INTRODUCTION

Crohn's disease (CD) is a chronic, nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear[1]. Since the 1950s, the incidence rate of CD has been steady-state growth of industrialization nations. CD is a common digestive disease with an incidence of 12.7/100000 residents very year in Europe[2]. In the course of CD, there are different types of perianal diseases, including fistula, abscess, anal fissure, stricture and dermatophyte. These lesions may appear prior to or accompanied by CD intestinal symptoms and are factors affecting the prognosis of CD[3]. Anal fistula is the main complication of CD. Studies have shown that 15%-45% of CD patients have perianal lesions such as anal fistula[1].

Fistulizing anoperineal lesions represent a complex disease phenotype for which the treatment requires a multidisciplinary approach[4]. Modern medical concepts describe that patients with CD anal fistula should be treated with drugs first, and surgical treatment should be considered when necessary to control intestinal inflammation[5]. The main therapeutic drugs used are antibiotics, immunosuppressants, biological agents, *etc*[6-9]. In recent years, many studies have shown that the use of mesenchymal stem cells can be a new treatment for specific cases of complex fistulas[10,11]. In addition, some scholars have suggested other new treatments, such as hyperbaric oxygen therapy, as a potential adjuvant treatment for patients with inflammatory bowel diseases (IBDs)[12,13]. However, these new treatments have not been fully developed into routine and safe technical procedures. Major constraints on the development of update therapeutic schedules is obviously correlated with the short of preclinical security and effectiveness data. Up to now, an ideal animal model that can reproduce sustaining anal fistula and an inflamed rectal mucosa is needed.

The main purpose of this research is to improve the colitis induction method and develop a simple, reliable and reproducible fistula animal model to assess new treatment strategies.

MATERIALS AND METHODS

Animals and groups

Twenty male New Zealand rabbits, weighing about 2.0 kg, were chosen and numbered after weighing (the grouping methods are listed in Table 1). They were raised and placed under the condition of no special pathogen. The laboratory was clean, with good light and ventilation. The indoor temperature was controlled between 24 and 28 ℃, and the relative humidity was maintained between 50% and 70%, with 10-15 air changes per hour and 12 h of light each day. On the day before and the day of the operation, the rabbits were fed formula and drank freely. Cages of rabbits were disinfected and kept separate. Sufficient water and food were given. The rabbits were kept in cages for 7 d to adapt to the environment. The rabbits were weighed on the day of operation and then every 7 d. This study was approved by the ethics committee of Changzhou University.

For each procedure (enema, surgery or endoscopy), 1.5% pentobarbital sodium (3.5 mL/kg) was used for ear vein anaesthesia, and dyclonine hydrochloride mucilage was locally applied around the anus to reduce the pain associated with surgery.

Model induction process

Proctitis: A total of 100 mg/kg 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) was dissolved in 50% ethanol (the total volume of solution is shown in Table 1) and was used for the induction of CD[14,15]. After 7 d of adaptive feeding, the experimental rabbits were fasted for 48 h and injected with 1.5% pentobarbital sodium through the ear vein. After anaesthesia, the rabbits were administered enemas with a TNBS + ethanol mixture by a 5 mL syringe through a central venous catheter every week according to the dose in Table 1 and then injected with air in a section of approximately 0.5 mm in length to remove the drug adhering to the syringe and enema tube wall as much as possible. Then, the rabbits were assigned to intervention groups A, B or C, where group C was used as a control.

Perianal fistula: On the 7th day after enema with TNBS, an anal fistula was caused by a minor operation. in the state of anaesthesia, the rabbits was fixed supine. Their perianal hair was shaved, and the area was disinfected with iodophor solution and then smeared with dyclonine hydrochloride mucilage. The elastic surgical seton (rubber band, diameter = 1.2 mm), soaked with TNBS solution in advance, was inserted into the needle core. For the experimental group, the seton was placed 1 cm from the anal margin at the same site, and a straight needle with a rubber band was used to puncture the rectum and then remove the punctured tissue from the body. A needle holder was used to clip the rubber band from the outside of the anus through the whole tunnel to make the rubber band pass through the perianal puncture opening, and a thin thread was used to fix the rubber band to prevent slippage and anal congestion. The external orifice is approximately 1 cm from the anus, as shown in Figure 1A. The surgical loop must be released without any tension. Finally, after the operation, the rabbits were returned to the feeding room, where they were observed and their vital signs were monitored until they woke up.

After the operation, a 1-mL syringe was used to inject TNBS mixed solution (diluted with 5% TNBS and absolute ethanol 1:1, total volume of 200 μL) into the fistula. Different doses of TNBS mixed solution (Table 1) were infused into the intestine once a week for three weeks, three times in total. To determine the best surgical procedure and reproducibility, 3 rabbits were randomly selected from each of groups A and B every 7 d, and the fistula setons were removed for endoscopic ultrasonography (EUS) assessment to evaluate the lumen. By the 28th day, the setons of all rabbits were removed. The characteristics of the two intervention groups and the different stages of the study are summarized in Figure 1B.

Model assessment

Clinical assessment: Clinical observation included: (1) recording the changes in daily activity, food intake, stool characteristics and body weight of the experimental animals and determining the disease activity index (DAI) score (Supplementary Table 1)[16]; (2) recording the number of deaths of the experimental animals in each group every day; and (3) checking whether the operation seton existed every day. In autonomous shedding, the new seton was inserted into the primary lumen again.

Endoscopic assessment: The colon macroscopic damage index (CMDI) was used for endoscopic assessment[17]. The CMDI was assessed according to the criteria described in Supplemental Table 2. Before the start of the study (TNBS enema administration), we performed an endoscopic examination of the rabbits to determine that the colon before treatment was normal, and these results were not included in the final statistics. After the study, the first intestinal endoscopy was performed on the 7th day (the day of surgical seton insertion). Morphological damage to the intestinal wall after the first intestinal administration was observed and scored. Then, endoscopy was performed every 7 d, and intestinal injury was observed and recorded. The last endoscopy was performed 21 d after the first enema. Endoscopy and scoring were performed by two experienced gastroenterologists (19 and 22 years of experience in the diagnosis and treatment of IBD, respectively).

Table 1 The volume of 2,4,6-trinitrobenzene sulfonic acid mixture administered by enema in each group

	Day 1	Day 7	Day 14
Group A (*n* = 9)	4.0 mL	5.0 mL	6.0 mL
Group B (*n* = 9)	5.0 mL	5.0 mL	5.0 mL
Group C (*n* = 2)	-	-	-
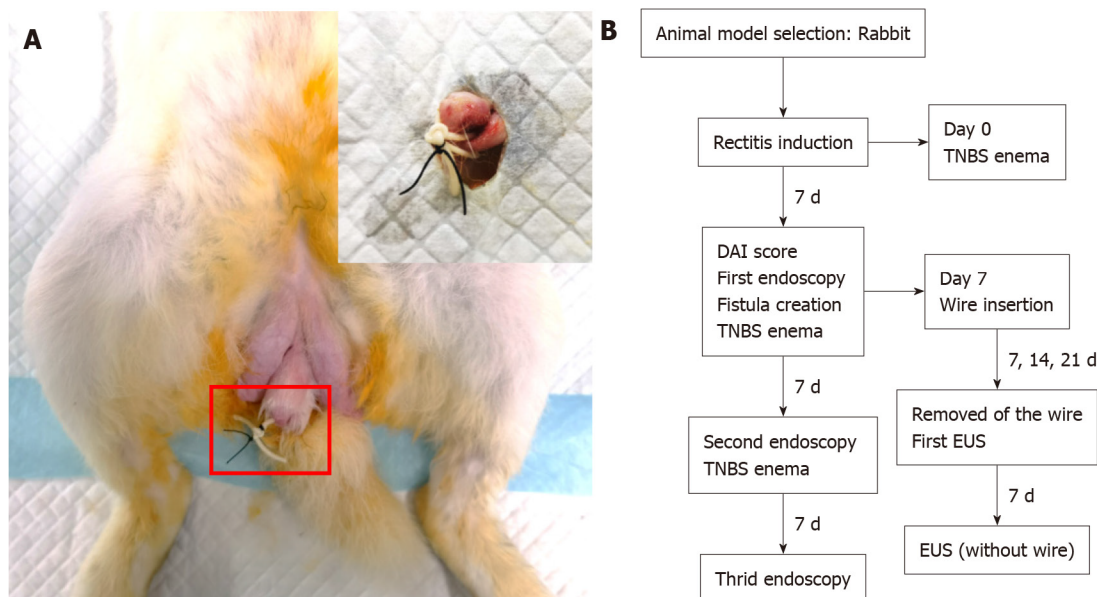
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Figure 1 Photograph of the external opening of the anal fistula, which was approximately 1 cm away from the anus, and a flowchart of the study protocol. A: The rubber band was used to hang the seton, and the leather band was fixed with a No. 0 operation seton to prevent slippage; B: One rabbit died one week after surgery. The remaining 17 rabbits were marked according to the length of insertion time. Three rabbits each were randomly selected from groups A and B. TNBS: 2,4,6-Trinitrobenzene sulfonic acid; DAI: Disease activity index; EUS: Endoscopic ultrasonography.

Histological examination: The tissue damage index (TDI) was used for the histological examination. The TDI was assessed using a modified version of the histological grading system described by MacPherson *et al*[18], as shown in Supplementary Table 3. At the same time as the endoscopic examination, 2-4 pieces of tissue with obvious inflammation and/or ulcers were clipped with biopsy forceps, fixed with neutral formaldehyde solution for 24 h, and stored at -4 ℃. Then, the specimens were embedded in paraffin, sliced continuously with a slicer, stained with haematoxylin-eosin, and finally scored histologically. Two experienced gastrointestinal pathologists performed blinded histological analyses.

The histological diagnosis of fistulas was ground on the following criteria: the internal orifice of the lumen is located on the rectal mucosa, and the external orifice of the lumen is located on the perineal skin. At the same time, it has the histological characteristics of proctitis (neutrophils, B and T lymphocytes, macrophages) were present. The feature of fistulas was decided by the presence or absence of epithelialization, fibration, and inflammation[19].

EUS assessment: The time of the insertion of the anal fistula operation thread was different in each group. After anal fistula formation, on the day of the removal of the thread inserted into the fistula, the perianal fistula of experimental rabbits in each group was examined by EUS for the first time, including mainly the observation of the fistula inner mouth, outer mouth, and course and the inflammation of the surrounding mucosa. The second EUS was performed on the 7th day after the removal of the thread. Spontaneous healing of the fistula was observed and recorded. Image recording and parameter interpretation were accomplished by a gastroenterologist (12 years of experience in diagnosis and treatment in IBD) and a ultrasound engineer (14 years of experience in interpreting ultrasound imaging). While they were blinded to groups of animals and histological results.

Statistical analysis

All the data were handled and analyzed by statistical software (SPSS 19.0), and the results are rendered as the mean ± SD. *P* < 0.05 was have been viewed as statistically critical.

RESULTS

Colitis model assessment

Clinical examination: In groups A and B, there were different degrees of loose stool and bloody stool visible to the naked eye. Rabbits ate less and were slow, low spirited, and occasionally irritable. Their weight gradually decreased with time. In group C, the body weight increased significantly with time, the activity was normal, and although there was occasional diarrhoea, there was no bloody stool. This condition was followed by the expected gradual weight recovery phase after the discontinuation of TNBS, which confirmed the healing of the colon injury. No rectal prolapse was observed. One week after the operation, the seton was removed from the perianal area of the experimental rabbits, and all the experimental rabbits showed two visible healing holes, which demonstrated the existence of the inner and outer holes. The rate of spontaneous seton shedding was approximately 17.6% (3/17) in each group. Also new setons were inserted again in the primary lumen of each animal. A total of 1 experimental rabbit died one week after surgery (group B) throughout the duration of experiments.

Endoscopy and pathology: Endoscopy was used to assess the modelling results. The process of colitis induction was smooth, and all rabbits underwent anal fistula surgery. One rabbit died one week after surgery. The rabbit was excluded from the results analysis. The remaining 17 rabbits were marked according to the length of insertion time.

After colitis was induced in the intervention group, the scores were determined, and the results are shown in Table 2. According to the statistical analysis (Table 2), except for the DAI score, the scores in group A were significantly higher than the scores in group B ($P < 0.05$).

In addition, we performed endoscopy in the process of colitis induction in rabbits of groups A and B and used biopsy forceps to obtain intestinal specimens for histological analysis. We found that although the rabbits in group B had obvious intestinal inflammation on the 7th day after the first TNBS enema, the intestinal inflammation at the last endoscopic examination was weaker than the intestinal inflammation in group A (Figures 2 and 3), showing that the inflammation of group A was higher than the inflammation of the other groups, and the modelling method of a TNBS dose increase in group A was better than the modelling method of other groups.

Model assessment of perianal fistulizing CD

EUS: All rabbits in groups A and B underwent two EUS scans of perianal fistulas. At the first EUS scan, all 17 rabbits (100%) had visible fistulas, also the external and internal orifice were noticeable in just about the greater part rabbits. At the second perianal EUS, that is, 7 d after the surgical seton was removed from the rabbits, the healing of the fistula in each group was different, as shown in Figure 4. Fistula was observed in 100% (6/6) of 6 rabbits with a surgical seton insertion time of 21 d. A scar was seen at the outer mouth of the fistulas, and granulation tissue hyperplasia was seen at the inner mouth. Other rabbits showed spontaneous healing of the fistula lumen and the disappearance of the fistula inner and outer orifices (Figure 4).

Pathology: Fibrosis has been distinguished in the connective tissue contiguous of the fistula 7 d after the insertion of the surgical seton. In addition, there were signs of acute (neutrophil infiltration and abscess formation) and chronic inflammation (lymphoplasmacyte infiltration, granuloma). On the 21st day after the insertion of the suture, granulation tissue was identified on the perianal orifice of the fistula. The pathologist positioned the rectal mucosa and thread through the anal sphincter <u>a</u>s the fistula,which shown in Figure 5.

Immunohistochemistry confirmed that group A was acute inflammation. Neutrophils and other inflammatory cells infiltrated in the fistula (Figure 6).

DISCUSSION

One of the most challenging phenotypes of CD is perianal fistula. The combination of perianal disease and CD predicts a significantly worse course[20]. The pathogenesis of CD and its complications are unclear. At present, there is no ideal curative treatment[21]. It is difficult to treat perianal fistulizing CD, which usually requires more active medical and surgical intervention.

Compared with rats, rabbits are mid- to large-sized animals. The rabbit anal and rectal anatomy is similar to that of humans and is of appropriate size[22,23]. The rabbit anatomy can maximize and tolerate the simulation of human perianal fistulizing CD-related auxiliary examinations, such as endoscopy, EUS, computed tomography, and magnetic resonance imaging (MRI). A preclinical model of rectal histological inflammation with perianal sphincteric fistula was established and observed continuously under endoscopy and confirmed by EUS. The diagnosis of fistula depends on EUS and histology.

As to the improvements in CD animal modelling through the use of this method involving TNBS[24], the method started with the administration of a small dose of TNBS, and then an increasing dose was

Table 2 Identification of the rabbit model of Crohn's disease induced by 2,4,6-trinitrobenzene sulfonic acid

	Group	Day 7	*P* value	Day 14	*P* value	Day 21	*P* value	Day 28	*P* value
DAI	A	4.22 ± 2.33	0.026	6.88 ± 0.93	0.020	8.00 ± 2.00	0.810	7.78 ± 1.56	0.021
	B	7.13 ± 2.53		8.75 ± 1.91		8.25 ± 2.25		5.75 ± 1.67	
Endoscopy scores	A	1.11 ± 0.78	0.002	2.44 ± 0.88	0.319	3.89 ± 0.93	< 0.001		
	B	2.88 ± 1.13		2.88 ± 0.83		1.63 ± 0.92			
Histology scores	A	1.44 ± 0.88	< 0.001	3.11 ± 0.93	0.017	4.11 ± 0.78	< 0.001		
	B	3.50 ± 0.93		2.00 ± 0.76		1.88 ± 0.99			

DAI: Disease activity index.
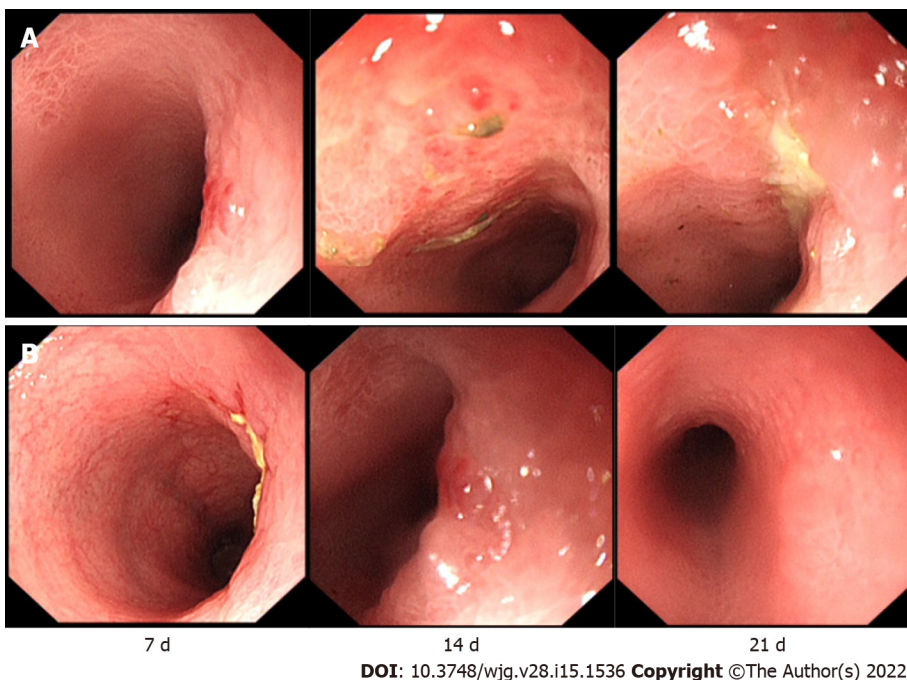
Figure 2 The rabbits in groups A and B underwent endoscopy 3 times. A: In group A, mild mucosal erosion was observed on the 7th day. On the 14th day, a large area of mucosal oedema and erosion appeared. On the 21st day, the surrounding mucosa was swollen, and a central ulcer had formed; B: In group B, ulceration occurred on the 7th day. On the 14th day, the mucosa was swollen, and the surface was hyperaemic and eroded. On the 21st day, the main manifestation was local congestion.

administered at the same intervals. This procedure not only simulated the characteristics human CD recurrence through repeated stimulation of the intestinal mucosa but also prolonged the duration of inflammation, showing that TNBS could induce a stronger inflammatory response by being administered at an increasing dose in a step-by-step manner. Group B showed improvements in bowel inflammation, possibly due to the initially established tolerable doses, but further investigation is needed. In our model, persistent inflammation is induced, and slight changes may occur with longer healing time, with histological changes replicating the unpredictability of CD. In the process of making an anal fistula, we used an elastic rubber band, which ensured the tension-free state of the fistula. At the end of the study, all rabbit models still had visible fistulas 7 d after the removal of the seton, and EUS showed continuous full-thickness high signal. However, the rabbits in which setons were inserted by 7 and 14 d had fistula healing, and 50% and 67% of the rabbits had fistulas visible on EUS after setons were removed 7 d later. Pathology revealed the same results for the longest seton insertion. The best option to obtain a preclinical model of perianal fistula consisted of low-dose incremental administration of TNBS and 21-d seton insertion. The induced fistula might has been described by mucosal ulcers extending to the perianal dermis. Pathologic examination showed augmented chronic submucosal infiltration of inflammatory cells, granuloma formation, and neutrophilic aggregation of colonic mucosa. At the same time, ultrasound endoscopy revealed continuous high signals in the skin around the anus, indicating the presence of an inflammatory response. These features are similar to those found in human perianal fistula CD and there is active inflammation in the fistula[19].

Figure 3 The rabbits in groups A and B underwent three consecutive endoscopic biopsies, and samples were stained with haematoxylin-eosin. A: Group A exhibited mild to moderate inflammation on day 7, manifested by fewer crypts, marked mucosal oedema, and minimal inflammatory cell infiltration; Group A exhibited fibrosis, hyperaemia, and moderate inflammatory cell infiltration on day 14, and severe inflammatory mucosal ulceration on day 21; B: On the 7th day, there was a higher number of shallow crypts, less oedema and less inflammatory cell infiltration in group B. On the 14th day, there was severe oedema, decreased crypts and less inflammatory cell infiltration in the mucosa, and on the 21st day, there was moderate fibrosis and oedema, mild inflammatory cell infiltration and a complete crypt structure (magnification ×100).

Perianal fistulizing CD models have been described differently by several authors. For example, the SAMP1/YitFc mouse model[25] or anal furunculosis model in canines[26] generate fistulas at random. A unprompted model similar to CD terminal ileitis was found in SAMP1/YitFc mice[25]. In this study, 5% of mice were found to form anal fistula naturally, which may be partially similar to human CD anal fistula. Whereas, only a small proportion of mice form fistula, which restricts application of this model in the study of CD. Size considerations make the murine model[27] more suitable for testing medical therapies than surgical interventions. For the canine model, considering the unpredictable onset time and the difficulty of inducing fistula, the applicability of the model is limited. A previous study developed another experimental model in 16 rabbits[28] by the surgical creation of a high transsphincteric fistula. Another study developed a pig anal fistula model[29] (6 pigs) to test a new biological plug, which was confirmed by histology. A recent article in 2019[19] described induced proctitis in rats by rectal enema using TNBS. Seven days later, the sphincter fistula was established using a surgical seton. In all these cited studies, unlike our research, previously published models either did not have proctitis or fistula inflammation, or a single high-dose enema caused high mortality. In addition, it is to some extent unethical that tissues can be obtained from these animals only after they are killed.

According to the current literature, we first applied endoscopy and EUS to the study of animal models of anal fistula in CD. Previous reports have used endoscopy to study oesophageal diseases in rabbits[30]. We performed colonoscopy with a small-diameter endoscope on rabbits, which can clearly show intestinal mucosa lesions. In addition, biopsy was performed in this mid- to large-sized animal for pathology assessment. In recent years, less invasive imaging modalities, such as pelvic MRI and EUS, have been used for fistula evaluation[31]. According to the latest available guidelines, contrast-enhanced pelvic MRI is generally considered the initial procedure for assessing perianal fistulizing CD[32]. However, EUS is considered a good alternative to MRI and has good accuracy when anal stenosis is excluded[32]. Although this method cannot obtain complete fistula tissue for histological analysis, we believe that, to a certain extent, this method is more in line with ethical requirements. Moreover, this simple, reproducible animal model can be used to evaluate the new treatment and efficacy of CD anal fistula.

With that in mind, we chose a small dose of TNBS as the initial stimulus and then repeated administration at the same intervals with dose increases. This procedure mimics to some extent the characteristics of human CD recurrence with repeated stimulation of the intestinal mucosa, prolongs the duration of inflammation, and imitates the process of CD recurrence. Second, the longest period of seton insertion was 21 d, an ideal result. Although in other studies, fistula-related thread insertion times were longer than ours[28],the prolongation of the seton insertion time may lead to longer fistula removal.

Figure 4 Visualization of a transsphincteric anal fistula *via* endoscopic ultrasonography. A: The internal and external openings of the experimental fistula can be directly observed; B: All rabbits with a 21-d insertion time of the surgical thread showed a complete fistula; C: The rabbits with short thread insertion times had different degrees of fistula healing or the disappearance of internal and external fistulas. D0: Day 0; D7: Day 7.

However, given that our study required continuous weekly bowel administration, extending the duration of the study increased the damage to the animals. Moreover, the criteria for the difference between the two experimental conditions (constant-dose or increased-dose TNBS) introduced in the study still need to be verified by larger animal experiments, and the molecular mechanism involved should be investigated. Thus, there are some limitations to this study.

CONCLUSION

In this study, a simple preclinical animal model of perianal fistulizing CD in rabbits was established by using an improved method of CD colitis induction. The model can simulate the human condition, and the intestinal and fistula lesions induced can be evaluated by EUS, endoscopic and histological examinations to assess new therapeutic strategies.
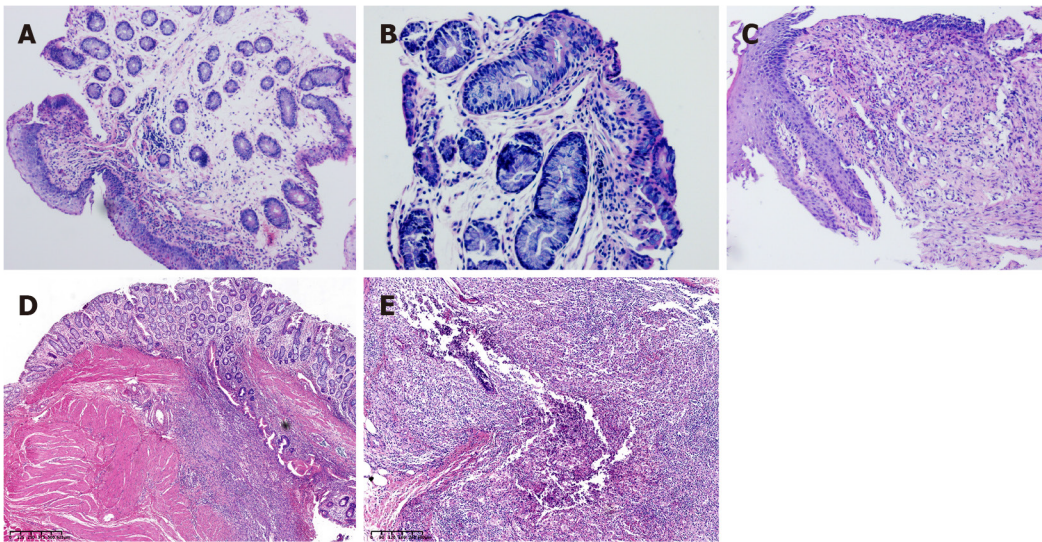
Figure 5 The histological characteristics of a fistula tract. A: Magnification ×100; B: Magnification ×200; C: Magnification ×100. The early histological changes of fistula are shown; D and E: Longitudinal sections; histological results (rabbits with setons inserted 21 d) showing the inflamed fistula tract. The fistula lumen is visible with internal (digestive side) and external (perineal skin with adipocytes) orifices. There were local inflammatory signs of suppurative inflammation with abscess formation around the fistula.
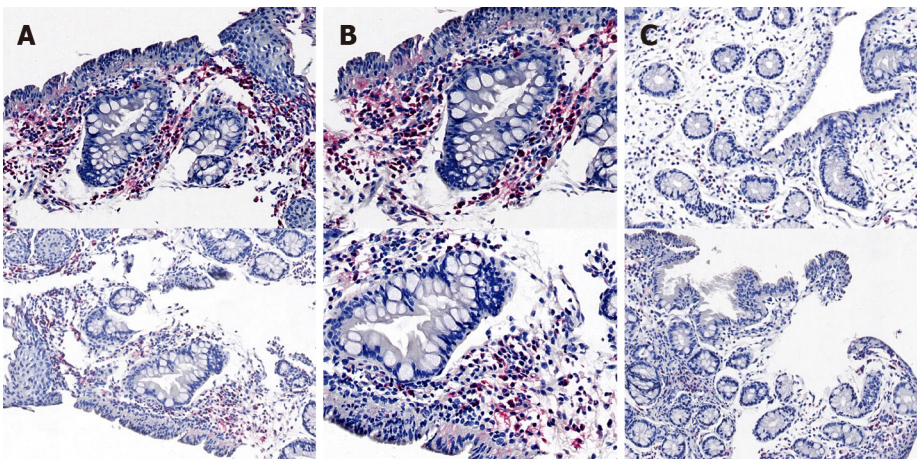
Figure 6 Immunohistochemistry of a fistula tract. A: Magnification ×10; B: Magnification ×20; C: Control. Immunohistochemistry of the fistula tract using anti-CD68 antibodies (upper panel) or anti-MPO antibodies (lower panel).

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is a chronic nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear. Anal fistula is the main complication of CD and is a difficult problem to solve at present. In recent years, there has been an increasing number of potential treatments for patients with inflammatory bowel diseases. However, these new treatments have not been fully developed into routine and safe technical procedures.

Research motivation

The main limitation in developing new therapies for CD with anal fistula is connected with the deficiency of preclinical safety and credible experimental data records. Therefore, an ideal animal model is needed to establish models of persistent anal fistula and an inflamed rectal mucosa.

Research objectives

The aim of this study was to improve the induction method of colitis and establish a reliable and reproducible perianal fistulizing CD animal model to evaluate new treatment strategies.

Research methods

Twenty male New Zealand rabbits underwent rectal enema with different doses of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce proctitis. Group A was treated with an improved equal interval small dose increasing method. The dosage of group B was constant. Seven days later, the rabbits underwent surgical creation of a transsphincteric fistula. Then, three rabbits were randomly selected each group every 7 d to remove the seton from the fistula. The rabbits were examined by endoscopy every 7 d, and biopsy forceps were used to obtain tissue samples from the obvious colon lesions for histological analysis. The disease activity index (DAI), colonoscopy and histological scores were recorded. Perianal endoscopic ultrasonography (EUS) was used to evaluate the healing of fistulas.

Research results

Except for the DAI score, the colonoscopy and histological scores in group A were significantly higher than those in the other groups ($P < 0.05$). In the ideal model rabbit group, on the 7th day after the removal of the seton, all animals had persistent lumens on EUS imaging, showing continuous full-thickness high signals. Acute and chronic inflammation, epithelialization, fibrosis, and peripheral proctitis of consecutive rectum are the histological features of fistula.

Research conclusions

A preclinical model of perianal fistulizing CD in rabbits was established by using an improved method of CD colitis induction. The model can simulate the human environment, and intestinal and fistula lesions can be evaluated by EUS, endoscopic and histological examinations to assess new therapeutic strategies.

Research perspectives

The establishment of a model of fistula associated with colitis allows the evaluation of different therapeutic approaches. However, fistula formation in animal models does not fully reflect the condition in humans. We hope that the simple, reliable and repeatable fistula animal model established by this improved colitis induction method can be used to evaluate new treatment strategies. The criteria for the difference between the two experimental conditions (constant-dose or increased-dose TNBS) introduced in the study still need to be verified by larger animal experiments, and the molecular mechanism involved should be investigated. The optimal animal model should include genetically mediated development of CD with anal fistula. However, an ideal model for preclinical research is difficult to establish due to the long experimental period required.

FOOTNOTES

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REFERENCES

1   **Schwartz DA**, Loftus EV Jr, Tremaine WJ, Panaccione R, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of fistulizing Crohn's disease in Olmsted County, Minnesota. *Gastroenterology* 2002; **122**: 875-880 [PMID: 11910338 DOI: 10.1053/gast.2002.32362]

2   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

3   **Hellers G**, Bergstrand O, Ewerth S, Holmström B. Occurrence and outcome after primary treatment of anal fistulae in Crohn's disease. *Gut* 1980; **21**: 525-527 [PMID: 7429313 DOI: 10.1136/gut.21.6.525]

4   **Hedin CRH**, Sonkoly E, Eberhardson M, Ståhle M. Inflammatory bowel disease and psoriasis: modernizing the multidisciplinary approach. *J Intern Med* 2021; **290**: 257-278 [PMID: 33942408 DOI: 10.1111/joim.13282]

5   **Bislenghi G**, Wolthuis A, Van Assche G, Vermeire S, Ferrante M, D'Hoore A. Cx601 (darvadstrocel) for the treatment of perianal fistulizing Crohn's disease. *Expert Opin Biol Ther* 2019; **19**: 607-616 [PMID: 31121104 DOI: 10.1080/14712598.2019.1623876]

6   **Herrlinger KR**, Stange EF. Twenty-five years of biologicals in IBD: What´s all the hype about? *J Intern Med* 2021; **290**: 806-825 [PMID: 34128571 DOI: 10.1111/joim.13345]

7   **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65 [PMID: 17241859 DOI: 10.1053/j.gastro.2006.11.041]

8   **Kamiński JP**, Zaghiyan K, Fleshner P. Increasing experience of ligation of the intersphincteric fistula tract for patients with Crohn's disease: what have we learned? *Colorectal Dis* 2017; **19**: 750-755 [PMID: 28371062 DOI: 10.1111/codi.13668]

9   **Gingold DS**, Murrell ZA, Fleshner PR. A prospective evaluation of the ligation of the intersphincteric tract procedure for complex anal fistula in patients with Crohn's disease. *Ann Surg* 2014; **260**: 1057-1061 [PMID: 24374520 DOI: 10.1097/SLA.0000000000000479]

10   **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016; **388**: 1281-1290 [PMID: 27477896 DOI: 10.1016/S0140-6736(16)31203-X]

11   **Ciccocioppo R**, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio C, Minelli A, Alvisi C, Vanoli A, Calliada F, Dionigi P, Perotti C, Locatelli F, Corazza GR. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut* 2011; **60**: 788-798 [PMID: 21257987 DOI: 10.1136/gut.2010.214841]

12   **Dulai PS**, Gleeson MW, Taylor D, Holubar SD, Buckey JC, Siegel CA. Systematic review: The safety and efficacy of hyperbaric oxygen therapy for inflammatory bowel disease. *Aliment Pharmacol Ther* 2014; **39**: 1266-1275 [PMID: 24738651 DOI: 10.1111/apt.12753]

13   **Lansdorp CA**, Gecse KB, Buskens CJ, Löwenberg M, Stoker J, Bemelman WA, D'Haens GRAM, van Hulst RA. Hyperbaric oxygen therapy for the treatment of perianal fistulas in 20 patients with Crohn's disease. *Aliment Pharmacol Ther* 2021; **53**: 587-597 [PMID: 33326623 DOI: 10.1111/apt.16228]

14   **Shibata Y**, Taruishi M, Ashida T. Experimental ileitis in dogs and colitis in rats with trinitrobenzene sulfonic acid--colonoscopic and histopathologic studies. *Gastroenterol Jpn* 1993; **28**: 518-527 [PMID: 8375625 DOI: 10.1007/BF02776950]

15   **Mohammad Jafari R**, Shayesteh S, Ala M, Yousefi-Manesh H, Rashidian A, Hashemian SM, Sorouri M, Dehpour AR. Dapsone Ameliorates Colitis through TLR4/NF-kB Pathway in TNBS Induced Colitis Model in Rat. *Arch Med Res* 2021; **52**: 595-602 [PMID: 33814208 DOI: 10.1016/j.arcmed.2021.03.005]

16   **Best WR**, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; **70**: 439-444 [PMID: 1248701 DOI: 10.1016/0011-7471(68)90042-9]

17   **Wallace JL**, MacNaughton WK, Morris GP, Beck PL. Inhibition of leukotriene synthesis markedly accelerates healing in a rat model of inflammatory bowel disease. *Gastroenterology* 1989; **96**: 29-36 [PMID: 2535830 DOI: 10.1016/0016-5085(89)90760-9]

18   **MacPherson BR**, Pfeiffer CJ. Experimental production of diffuse colitis in rats. *Digestion* 1978; **17**: 135-150 [PMID: 627326 DOI: 10.1159/000198104]

19   **Flacs M**, Collard M, Doblas S, Zappa M, Cazals-Hatem D, Maggiori L, Panis Y, Treton X, Ogier-Denis E. Preclinical Model of Perianal Fistulizing Crohn's Disease. *Inflamm Bowel Dis* 2020; **26**: 687-696 [PMID: 31774918 DOI: 10.1093/ibd/izz288]

20   **Kotze PG**, Magro DO, Saab B, Saab MP, Pinheiro LV, Olandoski M, Ayrizono MLS, Martinez CAR, Coy CSR. Comparison of time until elective intestinal resection regarding previous anti-tumor necrosis factor exposure: a Brazilian study on patients with Crohn's disease. *Intest Res* 2018; **16**: 62-68 [PMID: 29422799 DOI: 10.5217/ir.2018.16.1.62]

21   **Rackovsky O**, Hirten R, Ungaro R, Colombel JF. Clinical updates on perianal fistulas in Crohn's disease. *Expert Rev Gastroenterol Hepatol* 2018; **12**: 597-605 [PMID: 29792734 DOI: 10.1080/17474124.2018.1480936]

22   **de la Portilla F**, López-Alonso M, Borrero JJ, Díaz-Pavón J, Gollonet JL, Palacios C, Vázquez-Monchul J, Sánchez-Gil JM. The rabbit as an animal model for proctology research: anatomical and histological description. *J Invest Surg* 2011; **24**: 134-137 [PMID: 21524180 DOI: 10.3109/08941939.2010.550668]

23   **Aungst MJ**, Fischer JR, Bonhage MR, Albright TS, Noel KA, Wright J. Rectovaginal fistula model in the New Zealand white rabbit. *Int Urogynecol J* 2010; **21**: 885-888 [PMID: 20186389 DOI: 10.1007/s00192-010-1118-0]

24   **Ferreira-Duarte M**, Rodrigues-Pinto T, Sousa T, Faria MA, Rocha MS, Menezes-Pinto D, Esteves-Monteiro M, Magro F, Dias-Pereira P, Duarte-Araújo M, Morato M. Interaction between the Renin-Angiotensin System and Enteric Neurotransmission Contributes to Colonic Dysmotility in the TNBS-Induced Model of Colitis. *Int J Mol Sci* 2021; **22** [PMID: 34063607 DOI: 10.3390/ijms22094836]

25   **Rivera-Nieves J**, Bamias G, Vidrich A, Marini M, Pizarro TT, McDuffie MJ, Moskaluk CA, Cohn SM, Cominelli F. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. *Gastroenterology* 2003; **124**: 972-982 [PMID: 12671894 DOI: 10.1053/gast.2003.50148]

26   **Ferrer L**, Kimbrel EA, Lam A, Falk EB, Zewe C, Juopperi T, Lanza R, Hoffman A. Treatment of perianal fistulas with human embryonic stem cell-derived mesenchymal stem cells: a canine model of human fistulizing Crohn's disease. *Regen Med* 2016; **11**: 33-43 [PMID: 26387424 DOI: 10.2217/rme.15.69]

27   **Bruckner RS**, Nissim-Eliraz E, Marsiano N, Nir E, Shemesh H, Leutenegger M, Gottier C, Lang S, Spalinger MR, Leibl S, Rogler G, Yagel S, Scharl M, Shpigel NY. Transplantation of Human Intestine Into the Mouse: A Novel Platform for Study of Inflammatory Enterocutaneous Fistulas. *J Crohns Colitis* 2019; **13**: 798-806 [PMID: 30590414 DOI: 10.1093/ecco-jcc/jjy226]

28   **Benlice C**, Yildiz M, Baghaki S, Erguner I, Olgun DC, Batur S, Erdamar S, Ambarcioglu P, Hamzaoglu I, Karahasanoglu T, Baca B. Fistula tract curettage and the use of biological dermal plugs improve high transsphincteric fistula healing in an animal model. *Int J Colorectal Dis* 2016; **31**: 291-299 [PMID: 26310797 DOI: 10.1007/s00384-015-2374-8]

29   **Dryden GW**, Boland E, Yajnik V, Williams S. Comparison of Stromal Vascular Fraction with or Without a Novel Bioscaffold to Fibrin Glue in a Porcine Model of Mechanically Induced Anorectal Fistula. *Inflamm Bowel Dis* 2017; **23**: 1962-1971 [PMID: 28945635 DOI: 10.1097/MIB.0000000000001254]

30   **Huang J**, Shuang J, Xiong G, Wang X, Zhang Y, Tang X, Fan Z, Shen Y, Song H, Liu Z. Establishing a rabbit model of malignant esophagostenosis using the endoscopic implantation technique for studies on stent innovation. *J Transl Med* 2014; **12**: 40 [PMID: 24507720 DOI: 10.1186/1479-5876-12-40]

31   **Lahat A**, Assulin Y, Beer-Gabel M, Chowers Y. Endoscopic ultrasound for perianal Crohn's disease: disease and fistula characteristics, and impact on therapy. *J Crohns Colitis* 2012; **6**: 311-316 [PMID: 22405167 DOI: 10.1016/j.crohns.2011.09.001]

32   **Gecse KB**, Bemelman W, Kamm MA, Stoker J, Khanna R, Ng SC, Panés J, van Assche G, Liu Z, Hart A, Levesque BG, D'Haens G; World Gastroenterology Organization, International Organisation for Inflammatory Bowel Diseases IOIBD, European Society of Coloproctology and Robarts Clinical Trials; World Gastroenterology Organization International Organisation for Inflammatory Bowel Diseases IOIBD European Society of Coloproctology and Robarts Clinical Trials. A global consensus on the classification, diagnosis and multidisciplinary treatment of perianal fistulising Crohn's disease. *Gut* 2014; **63**: 1381-1392 [PMID: 24951257 DOI: 10.1136/gutjnl-2013-306709]

ORIGINAL ARTICLE

Case Control Study

Reevaluation of the expanded indications in undifferentiated early gastric cancer for endoscopic submucosal dissection

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Abstract

BACKGROUND

Although the criteria for the indication of endoscopic submucosal dissection (ESD) for undifferentiated early gastric cancer (UD-EGC) have been recently proposed, accumulating reports on the non-negligible rate of lymph node metastasis (LNM) after indicated ESD raise questions on the reliability of the current criteria.

AIM

To investigate the prevalence and risk factors of LNM in UD-EGC cases meeting the expanded indication for ESD.

METHODS

We retrospectively reviewed 4780 UD-EGC cases that underwent surgical resection between January 2008 and February 2019 at Asan Medical Center, a tertiary university hospital in Korea. To identify the risk factors of LNM of UD-EGC meeting the expanded criteria for ESD, we performed a case-control study by matching the cases with LNM to those without at a ratio of 1:4. We reviewed the clinical, endoscopic, and histologic features of the cases to identify features with a significant difference according to the presence of LNM. Univariate and

multivariate logistic regression analyses were performed to estimate the odds ratios (ORs).

RESULTS
Of the 4780 UD-EGC cases, 1240 (25.9%) were identified to meet the expanded indication for ESD. Of the 1240 cases, 14 (1.1%) cases had LNM. Among the various clinical, endoscopic, and histopathological features that were evaluated, mixed histology (tumors consisting of 10%-90% of signet ring cells) had a marginally significant association (P = 0.059) with the risk of LNM. Moreover, diffuse blurring of the muscularis mucosae (MM) underneath the tumorous epithelium, a previously unrecognized histologic feature, had a significant association with the absence of LNM (P = 0.028). Multivariate logistic regression analysis showed that the blurring of MM was the only explanatory variable significantly associated with a reduced risk of LNM (OR: 0.12, 95%CI: 0.02-0.95; P = 0.045).

CONCLUSION
The risk of LNM is higher than expected when using the current expanded indication for UD-EGC. Histological evaluation could provide useful clues for reducing the risk of LNM.

Key Words: Gastric cancer; Undifferentiated carcinoma; Endoscopic submucosal dissection; Lymph node metastasis

Core Tip: This was a retrospective study investigating the prevalence and risk factors of lymph node metastasis (LNM) in cases with undifferentiated early gastric cancer meeting the expanded indication for endoscopic submucosal dissection (ESD). We found that the incidence rate of LNM was 1.1% (14/1240), which was higher than expected for indicated ESD. A subsequent case-control study revealed that two histological features-histologic purity of tumors and blurring of the muscularis mucosae underneath the tumorous epithelium-are promising factors for predicting the risk of LNM. Combining these histologic features could improve the current expanded indication criteria for ESD.

INTRODUCTION

Endoscopic submucosal dissection (ESD) has gained popularity in the treatment of early gastric cancer (EGC) due to the benefits of organ preservation and maintenance of the quality of life. However, ESD cannot dissect lymph nodes around the stomach and diagnostic modalities such as endoscopic ultrasonography, computed tomography, and positron emission tomography cannot provide adequate data for detecting lymph node metastasis (LNM); as such, the indication for ESD for EGCs has been suggested based on the analysis of the risk of LNM in a large number of surgically resected specimens [1], and ESD is usually considered for tumors with a very low risk of LNM.

Undifferentiated EGC (UD-EGC) accounts for 40%-50% of EGCs, and has been reported to show a higher incidence of LNM than EGCs with differentiated histology[2,3]. Thus, gastrectomy with lymphadenectomy has been used as the standard treatment for UD-EGCs, and ESD for UD-EGC remains an investigational treatment[3,4]. In an attempt to expand the indication of ESD in UD-EGC, some researchers have reported that a select group of UD-EGCs had a very low possibility of LNM[1]. Accordingly, it has been suggested that ESD could be considered for UD-EGC cases when the tumor is an intramucosal lesion with a size of less than 2 cm and no sign of ulcer, and no further surgery is indicated when pathologic evaluation of the ESD specimen does not reveal lymphovascular invasion (LVI) or positive vertical and horizontal margin[1,4]. However, there have been several reports of lymph node or distant metastases arising after curative ESD in UD-EGC cases meeting the expanded criteria[5, 6]. These conflicting data raise a question on the reliability of the current expanded criteria for UD-EGC, and necessitates further efforts to identify more clinicopathologic features associated with the risk of LNM in UD-EGC.

Because LNM-negative patients can be curatively treated with minimally invasive ESD, evaluating the risk factor of LNM is crucial for determining the appropriate treatment. Although many studies have been performed to identify the clinicopathological factors associated with LNM in UD-EGC[7,8], the only risk factors that were identified include tumor size, depth of invasion, presence of LVI, and ulcer. Therefore, to obtain clarity regarding the treatment of UD-EGC, we investigated the risk of LNM of UD-EGCs meeting the criteria for the expanded indication for ESD, and performed a case-control study to identify the clinical, endoscopic, and histopathologic features related to the risk of LNM.

MATERIALS AND METHODS

Patients
We retrospectively reviewed the medical records of all patients who underwent curative gastrectomy with extended lymphadenectomy for UD-EGC at Asan Medical Center between January 2008 and February 2019. To focus on the histologic types that are most frequently encountered in clinical practice, we only included tumors diagnosed as "adenocarcinoma, poorly differentiated (with or without signet ring cell component)," "poorly cohesive carcinoma," and "signet ring cell carcinoma (SRCC)," and excluded rare variants such as mucinous adenocarcinoma and gastric carcinoma with lymphoid stroma. We also excluded patients with multiple tumors, tumors in the remnant stomach, any synchronous malignancy in other organs, a history of preoperative treatment such as ESD, and those who received neoadjuvant chemotherapy. Cases with less than 15 lymph nodes harvested were also excluded. Based on the original pathology reports of the remaining cases, we included those meeting all of the following criteria for the expanded indication of ESD: (1) confinement to the mucosal layer (pT1a); (2) size ≤ 2 cm; (3) absence of ulcer; and (4) absence of LVI[3,4].

To identify the clinical, endoscopic, and pathologic findings associated with the risk of LNM, we performed a case-control study by matching the patients with LNM to those without at a ratio of 1:4 in terms of sex, age at gastrectomy (± 2 years), and tumor size. Histologic review was conducted to confirm whether the cases indeed satisfy the expanded criteria. The selection process for our study population is shown as a flowchart (Figure 1).

Data collection
Clinical data, endoscopic features, and pathological characteristics of the study patients were evaluated. Endoscopic characteristics (*e.g.*, tumor location, macroscopic type of the lesion, endoscopic presence of ulcer, converging folds, exudates, and tumor island) were evaluated by two endoscopists (JY and KDC); the endoscopists independently reviewed the initial endoscopic images obtained before biopsy, and discussed with each other until a consensus was reached. The tumor locations were specified both longitudinally (upper *vs* middle *vs* lower third) and cross-sectionally (lesser curvature *vs* posterior wall *vs* greater curvature *vs* anterior wall). By referring to the classification system of the Japanese Research Society for Gastric Cancer[3], the macroscopic type of the lesion was evaluated based on the predominant type into three categories as follows: elevated type (including the protruded type and superficial elevated type), flat type (the superficial flat type), and depressed type (the superficial depressed type and excavated type)[3]. Endoscopic ulcer was defined as the presence of a mucosal defect of ≥ 3 mm. Converging folds were indicated by the presence of any centripetal folds in the EGC lesions. The representative endoscopic appearance is depicted in Supplementary Figure 1.

Histologic evaluation of the tumor and background stomach
Hematoxylin and eosin (HE)-stained glass slides produced at the time of initial diagnosis were evaluated to double-check the size of tumors in the greatest dimension, depth of invasion, and the status of lymph node metastasis. Entire tumors and adjacent non-tumor areas were serially sectioned at 3-to-4-millimeter intervals and made into paraffin blocks, which were then used to generate slides for histologic evaluation. Histologic mapping was performed to explicitly measure the size of a tumor, and the entirely embedded tumor and adjacent normal area were examined for the percentage of signet ring cells (SRCs), and status of background gastric mucosa. The percentage of SRCs was evaluated by examining the entire tumor sections. Definition of SRCs was established by the agreement between two pathologists (SYY and YSP) based on a recent consensus guideline on poorly cohesive gastric carcinoma [9]; according to the guideline, tumors almost exclusively (≥ 90%) consisting of SRCs were designated as SRCC, and those with < 10% of SRC components as poorly differentiated carcinomas (PDs), which encompass both poorly differentiated adenocarcinomas and non-SRC type of poorly cohesive carcinomas. The remaining tumors in which SRCs comprise 10%-90% of the components were designated as mixed tumors (*e.g.*, adenocarcinoma, poorly differentiated with SRC component and adenocarcinoma, moderately differentiated with SRCC). The status of background gastric mucosa was evaluated based on the likelihood of *Helicobacter pylori* (*H. pylori*) colonization as follows: less likely [minimal to mild chronic gastritis (CG) with no intestinal metaplasia (IM)], indeterminate (moderate CG or presence of IM), possible [chronic active gastritis (CAG)], and definite (CAG with clearly visible *H. pylori*). For each case, the section that seemed most likely to harbor *H. pylori* was selected for immuno-
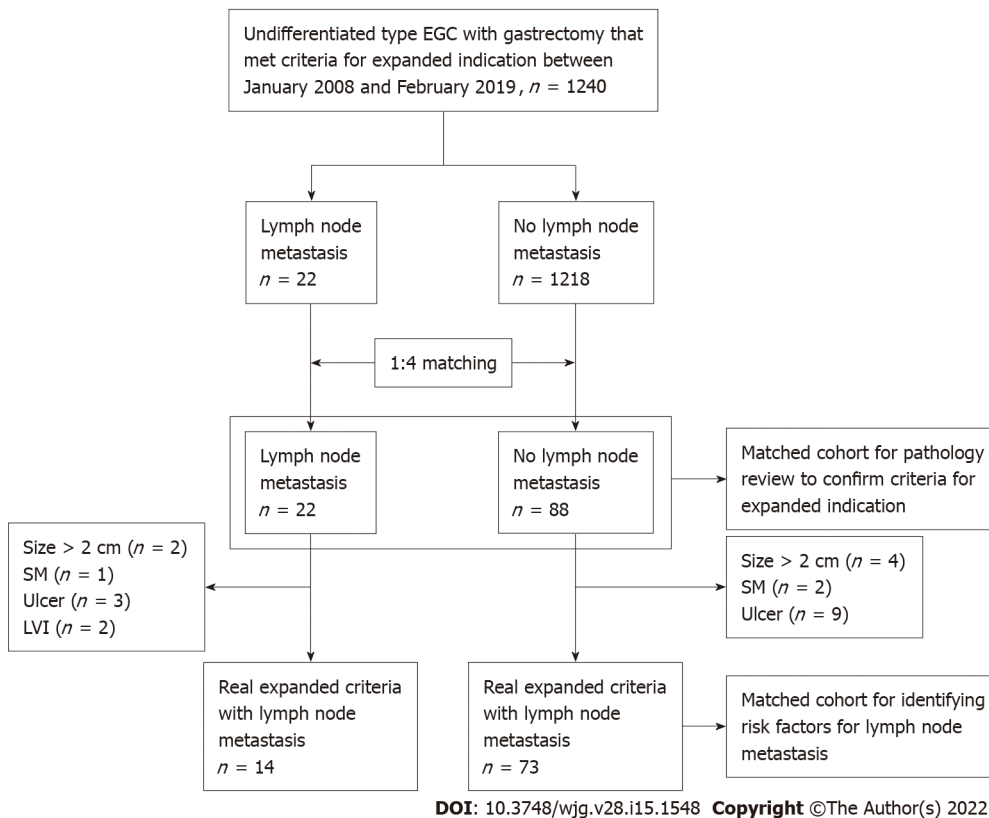
Figure 1 Flow chart of study patients. EGC: Early gastric cancer; SM: Submucosa; LVI: Lymphovascular invasion.

histochemical studies.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm-thick serial tissue sections of formalin-fixed paraffin-embedded (FFPE) blocks. For *H. pylori* evaluation, tissue sections were stained using the antibody against *H. pylori* (1:500, rabbit polyclonal, catalog No. 215A-76, Cell Marque, Rocklin, CA) using the OptiView DAB IHC Detection Kit on the BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, United States) according to the manufacturer's instructions. The abundance of immunohistochemically highlighted *H. pylori* was evaluated based on the Sydney system [10] as follows: 0 (absent), 1+ (*H. pylori* occupies < 1/3 of mucosa), 2+ (*H. pylori* occupies 1/3–2/3 of mucosa) and 3+ (*H. pylori* occupies > 2/3 of mucosa). *H. pylori* stain could not be performed in two cases due to the unavailability of FFPE blocks. TP53 staining was performed on representative sections at the time of initial diagnosis following the same protocol described above (1:1000, mouse monoclonal, clone DO-7, catalog No. M7001, Dako, Glostrup, Denmark). The degree of TP53 nuclear immunoreactivity was graded as 0 (no positive cells), 1+ (focal, faint positivity), 2+ (focal, moderate positivity), and 3+ (unanimously strong positivity); 0 was interpreted as the loss of expression, 1+ and 2+ as wildtype pattern, and 3+ as overexpression. TP53 status could not be evaluated in four cases due to the loss of stained slides.

Histologic evaluation of the tumor microenvironment

The abundance of tumor-infiltrating lymphocytes (TILs) was evaluated in HE slides in a semi-quantitative manner according to the proposed standardized methodology described in a recent consensus guideline[11].

The degree of peritumoral fibrosis was evaluated by Masson's trichrome (MT) staining. For 85 cases with accessible FFPE blocks, two sections per case-the deepest section and the edge of the tumor-were selected, and MT stain was performed on 4 μm-thick serial tissue sections of FFPE blocks using Trichome III Blue Staining Kit (Ventana Medical Systems) at BenchMark Special Stains platform (Ventana Medical Systems). The degree of fibrosis was visually graded as mild, moderate, or marked (Supplementary Figure 2C). For computer-aided image analysis, slides were scanned by the Pannoramic 250 Flash slide scanner (3D HISTECH, Budapest, Hungary) at 20× magnification with a resolution of 0.22 μm per pixel.

The degree of MT staining was quantified by pixel classification functionality of QuPath, an open-source software for analyzing digital pathology images (Supplementary Figure 2D)[12]. Briefly, the interface between MM and submucosa underneath the tumorous epithelium was manually annotated as

the region of interest (ROI). Two different types of pixel classifiers were trained and sequentially applied. The first classifier was trained to classify the ROI into empty (empty space and fat vacuoles) and non-empty areas. Non-empty areas were placed into the second classifier that graded the intensity of MT as 0 (vessel and MM), 1+ (mild), 2+ (moderate), or 3+ (marked). To express the intensity and extent of MT staining, a metric named "fibrosis score" was defined as follows:

$$\text{Fibrosis score} = \frac{3 \times \text{Area}_{3+} + 2 \times \text{Area}_{2+} + 1 \times \text{Area}_{1+}}{\text{Area of total non-empty area}}$$

Evaluation of blurring of muscularis mucosa

Blurring of MM underneath tumorous epithelium was primarily evaluated at scanning magnification. A case was judged to have blurred MM when any of the two MT-stained slides showed a focus of blurring or disruption of MM by fibrosis that caused loss of integrity relative to adjacent MM underneath the non-tumorous epithelium. If a case had more than one of such foci or the disruption was prominent enough to localize the tumor at scanning magnification, the case was judged to show diffuse blurring of MM (Figure 2A and B). At the foci of MM blurring, the distance from the invasive front to MM was measured. The value of 0 was assigned for tumors touching or invading into the MM. The interobserver reproducibility of MM blurring (non-diffuse or diffuse) was assessed by independent assessment of two pathologists (SYY and YSP). MM blurring assessed by SYY was used for subsequent statistical analysis.

Statistical analysis

Categorical variables are expressed as numbers with percentages and continuous variables are expressed as medians with interquartile ranges (IQRs). The Chi-squared test or Fisher's exact test was used to compare categorical variables as appropriate, and the *t*-test or Wilcoxon rank-sum test was used to compare continuous variables depending on the result of the Shapiro–Wilk normality test. Univariable and multivariable logistic regression analyses were performed to identify the risk factors by estimating the ORs and 95%CIs. Cohen's kappa was computed as a metric of interobserver reproducibility of MM blurring. P values < 0.05 were considered statistically significant. Statistical evaluations were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, United States) and R version 3.6.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria). The statistical methods of this study were reviewed by Kim HJ from the Department of Clinical Epidemiology and Biostatistics at Asan Medical Center, University of Ulsan College of Medicine.

RESULTS

Risk of LNM in UD-EGCs meeting the criteria for expanded indication for ESD

During the study period, 4780 patients underwent curative gastrectomy with extended lymphadenectomy for EGCs whose histology showed SRCs, PD, or a mixed type of both tumors. Of the 4780 patients, 1240 satisfied the criteria for the expanded indication for ESD. Among them, 22 patients had LNM and the remaining 1218 patients did not.

To identify the risk factors of LNM in patients with UD-EGC satisfying the expanded indication, a 1:4 case-control study was designed. According to the matching conditions, 22 patients with LNM were matched to 88 patients without. Subsequent pathology review was conducted for case and control patients to ensure that they satisfied the criteria for the expanded indication; as a result, 8 cases in the case group revealed histologic features inconsistent with the original pathology report and did not meet the criteria for the expanded indication as follows: size > 2 cm (n = 2), presence of submucosal invasion (n = 1), ulcer (n = 3), and LVI (n = 2). Similarly, 15 cases in the control group were excluded from the study owing to the following discrepancies from the original pathology report: size > 2 cm (n = 4), presence of submucosal invasion (n = 2), and ulcer (n = 9). Consequently, 14 (1.1%) patients among 1240 patients with UD-EGC were designated to have LNM and were included in the case group (LNM+), and 73 patients without LNM were included in the control group (LNM-).

Clinical and endoscopic features of the study patients

Clinical and endoscopic features of the 87 UD-EGC cases are summarized in Table 1. The median tumor size of the LNM+ group was 1.5 cm (IQR 1.3-1.8 cm), and the size of 11 (78.6%) LNM+ lesions exceeded 1 cm. Ten (71.4%) patients showed macroscopically depressed morphology, and the median number of harvested lymph nodes in the LNM+ group was 30.5. There were no significant differences between the LNM- and LNM+ groups in terms of the tumor location, gross type, and the number of retrieved LNs. Also, no significant differences were noted between the two groups in the preoperative endoscopic findings such as exudate, endoscopic ulcer, converging fold, and tumor island.

Detailed information on the 14 LNM+ cases is presented in Table 2; of those, cases 1, 3, 5, and 9 did not show endoscopic findings such as exudate, mucosal break, converging fold, and tumor island (Figure 3). Case 5 had six lymph node metastasis, but the endoscopic findings showed only a flat lesion with hyperemic mucosa (Figure 3C).

Table 1 Clinical and endoscopic features of the patients according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (*n* = 14)	*P* value
Age at diagnosis, yr (median, IQR)	47.0 (41.0-52.0)	43.5 (37.0-51.0)	0.276
Lesion size, cm (median, IQR)	1.5 (1.2-1.7)	1.5 (1.3-1.8)	0.485
Male, *n* (%)	25 (34.2)	4 (28.6)	0.918
Longitudinal location, *n* (%)			0.269
Upper	4 (5.5)	0 (0.0)	
Middle	22 (30.1)	2 (14.3)	
Lower	47 (64.4)	12 (85.7)	
Cross-sectional circumference, *n* (%)			0.421
Anterior wall	16 (21.9)	6 (42.9)	
Great curvature	16 (21.9)	2 (14.3)	
Posterior wall	23 (31.5)	3 (21.4)	
Lesser curvature	18 (24.7)	3 (21.4)	
Gross type, *n* (%)			0.440
Depressed	42 (57.5)	10 (71.4)	
Flat	25 (34.2)	4 (28.6)	
Elevated	6 (8.2)	0 (0.0)	
Number of retrieved LNs (median, IQR)	30.0 (25.0-37.0)	30.5 (25.5-37.0)	0.862
Endoscopic appearances, *n* (%)			
Exudate	6 (8.2)	1 (7.1)	> 0.999
Endoscopic ulcer	30 (41.1)	7 (50.0)	0.747
Converging fold	11 (15.1)	1 (7.1)	0.715
Tumor island	14 (19.2)	3 (21.4)	> 0.999

LNM: Lymph node metastasis; IQR: Interquartile range.

Histologic features of tumor and background stomach

Histopathologic features of the study patients and their background gastric mucosa were evaluated from HE stains and immunohistochemical stains for *H. pylori* and TP53 (Table 3). Except for the diagnostic category according to the proportion of SRCs, none of the histologic features showed significant differences between the LNM- and LNM+ groups. Although no significant difference was noted in the average percentage of SRCs, the LNM+ group tended to have more patients with mixed histology (consisting of 10–90% of SRCs) than pure SRCC or PD carcinoma (*P* = 0.059).

Histologic features of the tumor microenvironment

We further evaluated histologic features of the tumor microenvironment, abundance of TILs, and degree of peritumoral fibrosis (Table 4). We specifically focused on fibrosis because while evaluating histologic features of tumors and the background stomach, we observed that some tumors showed marked peritumoral fibrosis (Supplementary Figure 2A and B). We performed MT staining and analyzed the slides visually and computationally to investigate the degree, extent, and pattern of peritumoral fibrosis (Supplementary Figure 2C-E). However, neither TIL abundance nor the pattern and degree of peritumoral fibrosis showed a statistically significant association with the risk of LNM.

Blurring of MM as an independent risk factor for LNM

During the evaluation of MT stain, we noticed a peculiar pattern of fibrosis disrupting the MM, which could be categorized into diffuse and non-diffuse (focal plus no disruption) types (Figure 2). Importantly, the diffuse blurring of MM (Figure 2A and B) was significantly associated with the invasion of MM, shorter distance between the invasive front and MM, and higher fibrosis score (all *P* < 0.001, Supplementary Table 1). Some cases that lacked diffuse MM blurring had substantial peritumoral fibrosis (Supplementary Figure 3A) or invading MM (Supplementary Figure 3B-C), and other cases showed diffuse blurring of MM while being confined to the lamina propria or devoid of peritumoral

Table 2 Detailed clinical information of the 14 patients with lymph node metastasis

Case No.	Age (yr)	Sex	Type	Size (cm)	Location	Histology	Depth of invasion	Total number of dissected LNs	Number of metastatic LNs
1	59	Female	IIb	1.5	Middle	PD with SRC	LP	31	3
2	41	Female	IIc	1.3	Lower	PD with SRC	LP	18	3
3	57	Male	IIc	1.3	Lower	SRC	LP	21	1
4	47	Female	IIc	1.8	Middle	PD with SRC	LP	21	3
5	46	Female	IIb	2.0	Lower	PD with SRC	LP	25	6
6	37	Female	IIc	1.5	Lower	PD with SRC	MM	31	3
7	48	Female	IIc	1.5	Lower	PD with SRC	LP	37	1
8	35	Male	IIc	0.9	Lower	PD with SRC	LP	38	1
9	35	Female	IIc	1.5	Lower	PD with SRC	LP	27	1
10	52	Female	IIb	0.7	Lower	PD with SRC	LP	29	1
11	60	Male	III	0.6	Lower	PD with SRC	MM	30	1
12	39	Female	IIc	2.0	Lower	PD with SRC	LP	37	1
13	37	Female	IIb	1.8	Lower	PD with SRC	LP	42	1
14	33	Male	IIc	2.0	Lower	PD with SRC	LP	48	3

LNM: Lymph node metastasis; PD: Poorly differentiated carcinoma; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; LP: Lamina propria; MM: Muscularis mucosa.

fibrosis (Supplementary Figures 3D-E). Most importantly, we found a significant association between diffuse MM blurring and the absence of regional LNM (P = 0.028, Table 4). Multivariate logistic regression analysis with backward variable selection revealed that of the multiple clinical and histological variables, diffuse blurring of MM was the only statistically significant explanatory variable associated with the risk of LNM (OR: 0.12, 95%CI: 0.02–0.95; P = 0.045, Table 5).

Potential clinical utility of MM blurring

Independent assessment of MM blurring by a second pathologist revealed strong interobserver reproducibility with a Cohen's Kappa coefficient of 0.90 (95%CI: 0.80–0.96, Supplementary Table 2). Furthermore, MT staining on the ESD specimens of UD-EGC cases demonstrated that the presence of MM blurring could be readily evaluated in ESD specimens (Supplementary Figure 4). Collectively, these results suggest that MM blurring could serve as a feasible histologic marker that aids the decision on the follow-up strategy after ESD for UD-EGC cases meeting the expanded indication criteria.

DISCUSSION

In this study, we investigated the risk factors for LNM in patients with UD-EGC meeting the criteria for the expanded indication for ESD by using surgically resected cases. Our results demonstrated that the incidence rate of LNM in cases of UD-EGC meeting the criteria for the expanded indication for ESD was 1.1% (14/1240). By reviewing the clinical, endoscopic features, and pathologic results, we found that the LNM- and LNM+ groups did not show significant differences in terms of preoperative clinical and endoscopic features. On the other hand, histologic features such as mixed histology (P = 0.059) and blurring of MM (P = 0.028) showed a notable difference according to the presence of LNM, suggesting

Table 3 Histologic features of the tumors and background stomach according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (*n* = 14)	*P* value
Depth of invasion			0.503
LP	53 (72.6)	12 (85.7)	
MM	20 (27.4)	2 (14.3)	
Size, cm	1.5 (1.2-1.7)	1.5 (1.3-1.8)	0.642
% of SRCs			0.157
< 10%	17 (23.3)	0 (0)	
≥ 10% and < 50%	35 (47.9)	10 (71.4)	
≥ 50% and < 90%	14 (19.2)	3 (21.4)	
≥ 90%	7 (9.6)	1 (7.1)	
Diagnostic category according to the proportion of SRCs			0.059
Non-mixed (SRCC and PD)	24 (32.9)	1 (7.1)	
Mixed (PD with SRC component)	49 (67.1)	13 (92.9)	
Background stomach			0.278
Mild CG	8 (11.0)	0 (0)	
Moderate CG or IM	24 (32.9)	3 (21.4)	
CAG	22 (30.1)	4 (28.6)	
CAG with visible *H. pylori*	19 (26.0)	7 (50.0)	
H. pylori abundance, *n*/total *n*			0.263
0	23/71 (32.4)	2/14 (14.3)	
1+	14/71 (19.7)	3/14 (21.4)	
2+	14/71 (19.7)	6/14 (42.9)	
3+	20/71 (28.2)	3/14 (21.4)	
TP53 expression, *n*/total			> 0.999
Loss (0)	3/70 (4.3)	0/14 (0)	
Wildtype pattern (1+/2+)	63/70 (90.0)	13/14 (92.9)	
Overexpression (3+)	4/70 (5.7)	1/14 (7.1)	

LNM: Lymph node metastasis; IQR: Interquartile range; LP: Lamina propria; MM: Muscularis mucosa; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; PD: Poorly differentiated carcinoma encompassing adenocarcinoma and non-signet ring cell type of poorly cohesive carcinoma; CG: Chronic gastritis; IM: Intestinal metaplasia; CAG: Chronic active gastritis; *H. pylori*: *Helicobacter pylori*.

that histologic evaluation could be useful for improving patient stratification.

To date, ESD for UD-EGC has required an expanded indication, and surgery has been the standard treatment because the risk of LNM has been shown to be relatively higher in UD-EGCs than in differentiated EGCs, thus raising concern about the long-term outcomes. However, in the recent guidelines in Japan, UD-EGC lesions have been integrated into the absolute indication for ESD[13]. Li *et al*[14] reported that there were no cases of LNM in patients with UD-EGC meeting the expanded indications of ESD. Another study revealed that LNM was not found in intramucosal cancer when the lesion was 20 mm or less without LVI and ulcerative findings[1]. However, these studies have the limitations of small sample sizes and retrospective study design. In a recent multicenter clinical trial, Takizawa *et al*[15]. reported that patients who were followed after undergoing curative ESD for UD-EGC showed neither local/distant recurrence nor deaths due to gastric cancer, thereby suggesting favorable clinical outcomes of ESD for UD-EGC; however, this study had a single-arm design and the outcomes after ESD were not compared with those after surgery. In addition, other studies reported contrasting results in that up to 2.3% of patients with UD-EGC meeting the criteria of expanded indications (intramucosal cancer, size of ≤ 20 mm without LVI and ulcerative findings) were found to have LNM[16]. Our study also showed that 1.1% of 1240 patients meeting the criteria for the expanded indication for ESD showed LNM. Considering these conflicting results, further targeted investigations are required regarding the risk of

Table 4 Histologic features of the tumor microenvironment according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (*n* = 14)	*P* value
TIL abundance			0.438
< 10%	25 (34.2)	7 (50.0)	
10%-20%	33 (45.2)	6 (42.9)	
≥ 20%	15 (20.5)	1 (7.1)	
Degree of central fibrosis			0.522
Mild	6 (8.5)	2 (14.3)	
Moderate	35 (49.3)	8 (57.1)	
Marked	30 (42.3)	4 (28.6)	
Degree of peripheral fibrosis			
Mild	11 (15.5)	2 (14.3)	0.495
Moderate	53 (74.6)	9 (64.3)	
Marked	7 (9.9)	3 (21.4)	
Distribution of fibrosis			
Central = peripheral	32 (45.1)	7 (50.0)	0.204
Central > peripheral	33 (46.5)	4 (28.6)	
Central < peripheral	6 (8.5)	3 (21.4)	
Fibrosis score	0.55 (0.38-0.83)	0.53 (0.31-0.77)	0.273
Blurring of MM, *n*/total			0.028
Non-diffuse (absent/focal)	43/71 (60.6)	13/14 (92.9)	
Diffuse	28/71 (39.4)	1/14 (7.1)	

LNM: Lymph node metastasis; TIL: Tumor-infiltrating lymphocytes; IQR: Interquartile range; MM: Muscularis mucosa.

LNM in UD-EGC cases meeting the expanded criteria.

LNM is the most important factor for deciding the treatment strategy in cases of UD-EGC. For the appropriate usage of ESD in UD-EGC, the characteristics of UD-EGCs with LNM should be clarified so that such cases should be excluded from consideration for ESD. Hence, we reviewed the original pathologic reports of 4781 surgically resected UD-EGC and found that 1240 cases met the criteria for the expanded indication for ESD, 22 of whom exhibited regional LNM. However, a subsequent histologic review revealed that 8 of the 22 cases with LNM and 15 out of the matched 88 control cases did not satisfy the criteria for expanded indications due to deviation in size from the original pathology reports (*n* = 2 in the case group, *n* = 4 in the control group) and the presence of ulcer (*n* = 3 in the case group, *n* = 9 in the control group). The discrepancy likely occurred because unlike mucosectomy specimens, gastrectomy specimens do not mandatorily undergo systematic evaluation for the tumor size and the presence of an ulcer[3,17]. In our study, three cases with submucosal invasion that had been misdiagnosed as mucosal cancer showed deceptive histologic features that called for careful examination. In one case, it seemed that the site of submucosal invasion had been missed because the tumor was located in an extensively thick and undulating mucosa. The tumor in another case was accompanied by a massively lymphoid stroma so that the tumor cells in the submucosa were barely visible. Tumor cells of the remaining case were almost indistinguishable from macrophages. TP53 immunostaining, which is routinely performed for all stomach cancer cases at our institution, was useful in highlighting the tumor cells in the submucosa in the latter two cases. Careful attention is needed to diagnose ESD cases showing features of discrepant cases.

This discrepancy after the second histologic review implies a more serious message. Considering that the number of patients in the LNM+ group decreased from 22 to 14 after the second histologic review, the total number of UD-EGC patients satisfying the expanded criteria could decrease from 1240 if the entire case cohort was reviewed. As a consequence, the actual incidence rate can be actually higher than 1.1%. Therefore, our results suggest that UD-EGC cases meeting the criteria for the expanded indication does have a risk of LNM, which may be too higher to consider endoscopic resection. Therefore, further research is needed to discover clinical, endoscopic, and histologic features of UD-EGC that can aptly supplement the current expanded criteria.

**Table 5 Logistic regression analysis for the risk of Lymph node metastasis**

Variable	OR	95%CI	*P* value
Depth of invasion			
LP	1		
MM	0.44	0.09-2.15	0.312
Diagnostic category according to % of SRCs			
Non-mixed (SRC and PD)	1		
Mixed (PD with SRC component)	6.37	0.79-51.6	0.083
Background stomach			
CG	1		
CAG	2.86	0.74-11.1	0.129
Presence of *H. pylori*			
Absent (0)	1		
Present (≥ 1+)	2.88	0.59–13.9	0.190
TIL abundance			0.409
< 10%	1		
10%-20%	0.65	0.19-2.17	0.484
≥ 20%	0.24	0.03-2.13	0.199
Fibrosis score	0.34	0.05-2.49	0.285
Blurring of MM			
Non-diffuse (absent/focal)	1		
Diffuse	0.12	0.02-0.95	0.045

LNM: Lymph node metastasis; OR: Odds ratio; LP: Lamina propria; MM: Muscularis mucosa; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; PD: Poorly differentiated carcinoma encompassing adenocarcinoma and non-signet ring cell type of poorly cohesive carcinoma; CG: Chronic gastritis; CAG: Chronic active gastritis; TIL: Tumor-infiltrating lymphocytes; *H. pylori*: *Helicobacter pylori*.

For this purpose, the tumor characteristics of 14 patients with LNM were evaluated. The tumor characteristics including tumor location, gross type, the number of retrieved LNs, and endoscopic appearances did not have significant associations with LNM. According to histologic analysis, among various histologic features, mixed histology (consisting of 10%-90% SRCs) in comparison with non-mixed histology (*i.e.*, pure SRCC, poorly cohesive carcinoma, or poorly differentiated adenocarcinoma) was the only variable with a marginal statistical significance (*P* = 0.059). This is consistent with previous studies suggesting more aggressive biology of EGC by using mixed histology rather than pure adenocarcinoma and SRCC[18,19]. Therefore, it is likely that we would have reached statistical significance if a larger number of cases were analyzed.

Inspired by the recent interest in the role of the tumor microenvironment on metastasis[20], we further investigated the histologic features of the tumor microenvironment, especially the pattern and degree of peritumoral fibrosis. Previous studies on submucosal fibrosis of EGCs have mostly focused on its negative effect on successful ESD[21-25]. Conversely, we focused on whether the extent or pattern of submucosal fibrosis had an impact on LNM. While the degree of submucosal fibrosis did not show a significant association with LNM, we unexpectedly discovered a significant association between MM blurring and LNM. A structural study on the distribution of lymphatic and blood capillaries of human gastric mucosa showed that lymphatic capillaries were present in the deep lamina propria adjacent to and within the MM[26]. As such, we hypothesize that the blurring of MM is a consequence of an exaggerated anti-tumoral reaction against tumor cells trying to invade the lymphatics within the MM. In contrast with the traditional concept of the pro-tumorigenic role of fibrosis, recent studies have suggested that tumor-related fibrosis can also restrain cancer initiation, proliferation, and metastasis [27]. Therefore, it is possible that tumors that managed to elicit anti-tumoral fibrosis against the tumor cells' attempt to invade lymphatics are seen as having blurred MM, and are hence less likely to metastasize into the regional lymph nodes.

Considering the intimate relationship between lymphatics and the MM, it can be assumed that the tumor's proximity to the MM would be significantly associated with the risk of LNM. Indeed, it has
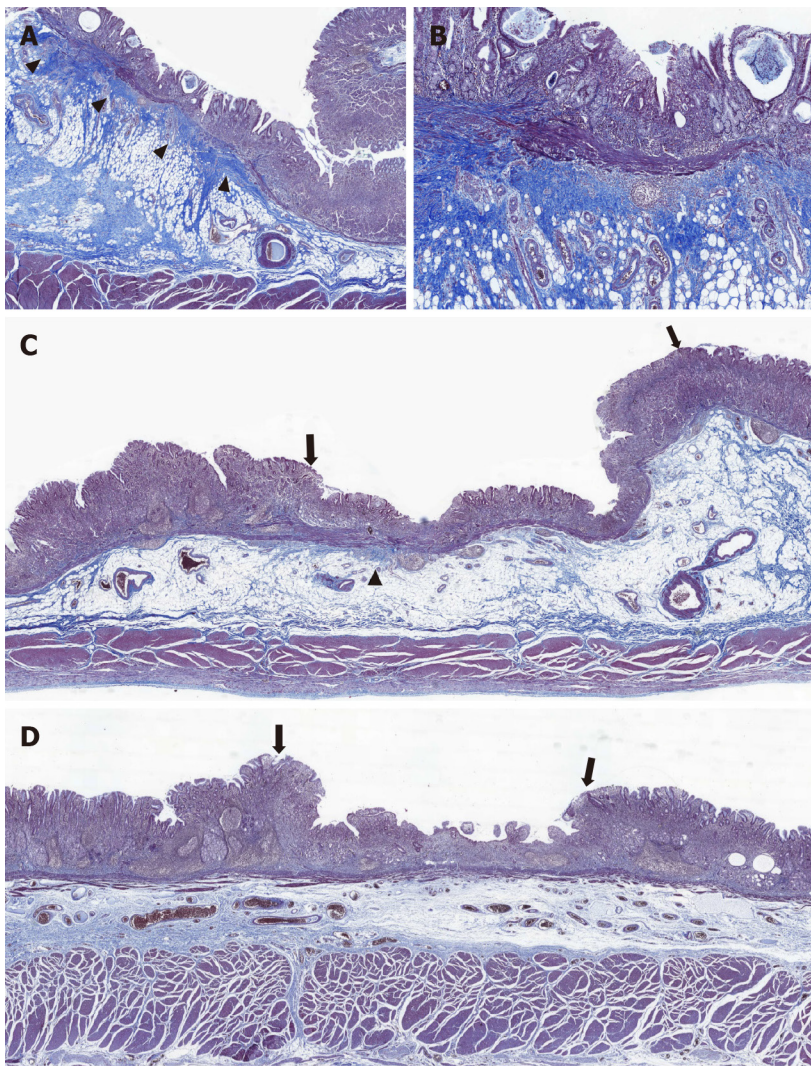
Figure 2 Blurring of muscularis mucosa underneath the tumorous epithelium. Representative images of tumors with diffuse, focal, and no blurring of muscularis mucosa. A: Diffuse blurring of muscularis mucosa (MM) was prominent enough to localize the tumor at scanning magnification (arrowhead); B: At higher magnification (40´), the thickness of MM appeared irregular due to collagen fibers disrupting the muscle fibers of MM; C: The majority of MM underneath the tumorous epithelium (both ends are marked by arrows) was undisrupted compared with adjacent MM underneath the non-tumorous epithelium, making the foci of MM blurring focal (arrowhead); D: With no blurring of MM, it was difficult to localize the tumor (both ends are marked by arrows) at scanning magnification based on the status of MM.

been reported that tumors invading the MM are more likely to metastasize into regional lymph nodes than those limited to the lamina propria[28]. However, we failed to reach the same conclusion in our current study, and our study might suggest the opposite conclusion considering the significant association between MM invasion and MM blurring. This may be due to the fact that our control group is biased toward tumors invading the MM; however, unlike the two previous studies, we only focused on UD-EGC cases meeting the expanded criteria for ESD.

We hypothesize that the seemingly counterintuitive result of our study might be explained by the differences in the mode of invasion between differentiated and undifferentiated GCs, considering the results of recent studies that elucidated the various modes of cancer cell invasion, ranging from single-cell migration to collective invasion[29]. Because poor differentiation often involves the loss of cellular adhesion molecules[30], undifferentiated GCs might preferentially invade as single cells. For this reason, the main body of poorly differentiated tumors does not necessarily need to be in close proximity to lymphatics to invade them, and desmoplasia is more likely attributable to anti-tumoral microenvironmental responses rather than invading tumor cells. On the other hand, differentiated GCs would invade the adjacent normal structures by forming glands. Glandular structures are likely more destructive than scattered cells, and massive violation of the normal structure itself is sufficient enough to elicit fibrosis; for this reason, desmoplasia in differentiated GCs would more reflect the invasiveness of the tumors than anti-tumoral responses. Further studies in independent cohorts are needed to validate the hypothesis on the differential significance of MM blurring in differentiated and undifferentiated GCs.
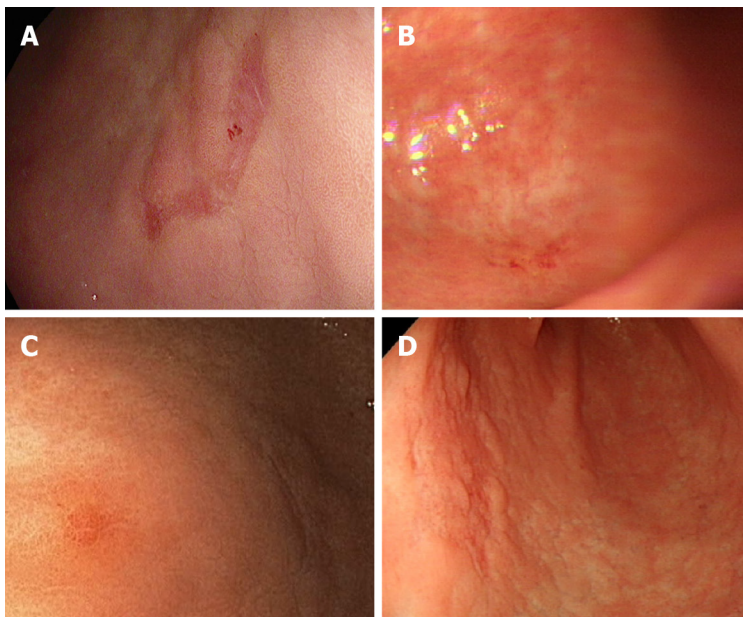
Figure 3 Endoscopic images of the cases with lymph node metastasis without exudate, mucosal break, converging fold, and tumor island. A: Case 1, B: Case 3, C: Case 5, D: Case 9.

There are several limitations to our study. First, because we excluded cases that showed deviation from the original pathology reports (*e.g.*, larger tumor size, ulcer, LVI, submucosal invasion), the study population was reduced and the case-control study could not be performed as originally intended. Second, the validity of MM blurring may benefit from further scrutiny; aside from the four tumors that were small enough to be embedded in single blocks, we examined two representative sections per each case and judged a case as having blurred MM when such foci were identified in any of the two sections. As such, it is possible that the cases classified as clear MM might have disrupted MM in unexamined sections. Finally, this study had limitations inherent to the nature of a retrospective, single-center study. Although the number of patients with LNM was small, this is because the incidence of LNM in patients with UD-EGC meeting the expanded indications of ESD is low. Considering the low incidence, a large-scale, prospective, multicenter study is needed to confirm our findings. Despite these limitations, we believe that our study appropriately highlights the fact that diffuse blurring of MM may be a potential predictive factor for the risk of LNM in cases of UD-EGC meeting the expanded criteria for ESD. Further research is needed to validate our results and to elucidate its mechanistic basis.

CONCLUSION

In conclusion, our results show that cases of UD-EGC meeting the criteria for the expanded indication have the risk for LNM, albeit low (1.1%), and that routine histological examination has practical limitations for identifying such cases. When ESD is planned for a case of UD-EGC, obtaining detailed informed consent after the disclosure of the risk of LNM is necessary, and careful observation is essential. A model for patient stratification based on histologic evaluation of the proportion of SRCs and MM blurring in ESD specimens could be useful for identifying the patients with a higher risk of LNM.

ARTICLE HIGHLIGHTS

Research background

There have been several reports of lymph node or distant metastases arising after curative endoscopic submucosal dissection (ESD) in undifferentiated early gastric cancer (UD-EGC) cases meeting the expanded criteria.

Research motivation

The clinicopathologic features associated with the risk of lymph node metastasis (LNM) in UD-EGC have not been well-studied.

Research objectives

To investigate the prevalence and risk factors of LNM in UD-EGC cases meeting the criteria for the expanded indication for ESD.

Research methods

In this retrospective study, we investigated the risk of LNM of UD-EGC meeting the criteria for the expanded indication for ESD, and performed a matched case-control study to identify the clinical, endoscopic, and histopathological features associated with the risk of LNM. Univariate and multivariate logistic regression analyses were performed to identify the risk factors by estimating the odds ratios.

Research results

The incidence rate of LNM in UD-EGC cases meeting the criteria for the expanded indication for ESD was 1.1% (14/1240). No significant differences existed between the LNM group and the matched non-LNM group in terms of preoperative clinical endoscopic features and conventional histologic features. In the tumor microenvironment, blurring of muscularis mucosa (MM) underneath the tumorous epithelium was associated with the risk of LNM.

Research conclusions

The risk of LNM was higher than expected when using the current expanded indication for UD-EGC. Evaluation of blurring of MM could provide useful clues for reducing the risk of LNM.

Research perspectives

Further studies are needed to validate the significance of MM blurring and elucidate its mechanistic basis. Eventually, an improved model for patient stratification based on detailed histologic evaluation of ESD specimens should be established to identify patients with a high risk of LNM.

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REFERENCES

1   **Gotoda T**, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, Kato Y. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. *Gastric Cancer* 2000; **3**: 219-225 [PMID: 11984739 DOI: 10.1007/pl00011720]

2   **Hong S**, Won YJ, Lee JJ, Jung KW, Kong HJ, Im JS, Seo HG; Community of Population-Based Regional Cancer Registries. Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2018. *Cancer Res Treat* 2021; **53**: 301-315 [PMID: 33735559 DOI: 10.4143/crt.2021.291]

3   **Japanese Gastric Cancer Association**. . Japanese gastric cancer treatment guidelines 2014 (ver. 4). *Gastric Cancer* 2017; **20**: 1-19 [PMID: 27342689 DOI: 10.1007/s10120-016-0622-4]

4   **Guideline Committee of the Korean Gastric Cancer Association (KGCA)**; Development Working Group & Review Panel. Korean Practice Guideline for Gastric Cancer 2018: an Evidence-based, Multi-disciplinary Approach. *J Gastric Cancer* 2019; **19**: 1-48 [PMID: 30944757 DOI: 10.5230/jgc.2019.19.e8]

5   **Kang HJ**, Kim DH, Jeon TY, Lee SH, Shin N, Chae SH, Kim GH, Song GA, Srivastava A, Park DY, Lauwers GY. Lymph node metastasis from intestinal-type early gastric cancer: experience in a single institution and reassessment of the extended criteria for endoscopic submucosal dissection. *Gastrointest Endosc* 2010; **72**: 508-515 [PMID: 20554277 DOI: 10.1016/j.gie.2010.03.1077]

6   **Zhu ZL**, Shi HP, Beeharry MK, Feng TN, Yan M, Yuan F, Zheng-Gang Zhu, Zhang BY, Wu W. Expanding the indication of endoscopic submucosal dissection for undifferentiated early gastric cancer is safe or not? *Asian J Surg* 2020; **43**: 526-531 [PMID: 31706922 DOI: 10.1016/j.asjsur.2019.08.006]

7   **Hu Q**, Dekusaah R, Cao S, Pang T, Wang Y, Zhang B, Lv Y, Zhang X, Ling T, Zhuge Y, Wang L, Zou X, Zhang W, Huang Q, Xu G. Risk Factors of Lymph Node Metastasis in Patients with Early Pure and Mixed Signet Ring Cell Gastric Carcinomas. *J Cancer* 2019; **10**: 1124-1131 [PMID: 30854120 DOI: 10.7150/jca.29245]

8   **Zhang Y**, Liu Y, Zhang J, Wu X, Ji X, Fu T, Li Z, Wu Q, Bu Z, Ji J. Construction and external validation of a nomogram that predicts lymph node metastasis in early gastric cancer patients using preoperative parameters. *Chin J Cancer Res* 2018; **30**: 623-632 [PMID: 30700931 DOI: 10.21147/j.issn.1000-9604.2018.06.07]

9   **Mariette C**, Carneiro F, Grabsch HI, van der Post RS, Allum W, de Manzoni G; European Chapter of International Gastric Cancer Association. Consensus on the pathological definition and classification of poorly cohesive gastric carcinoma. *Gastric Cancer* 2019; **22**: 1-9 [PMID: 30167905 DOI: 10.1007/s10120-018-0868-0]

10   **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181 [PMID: 8827022 DOI: 10.1097/00000478-199610000-00001]

11   **Hendry S**, Salgado R, Gevaert T, Russell PA, John T, Thapa B, Christie M, van de Vijver K, Estrada MV, Gonzalez-Ericsson PI, Sanders M, Solomon B, Solinas C, Van den Eynden GGGM, Allory Y, Preusser M, Hainfellner J, Pruneri G, Vingiani A, Demaria S, Symmans F, Nuciforo P, Comerma L, Thompson EA, Lakhani S, Kim SR, Schnitt S, Colpaert C, Sotiriou C, Scherer SJ, Ignatiadis M, Badve S, Pierce RH, Viale G, Sirtaine N, Penault-Llorca F, Sugie T, Fineberg S, Paik S, Srinivasan A, Richardson A, Wang Y, Chmielik E, Brock J, Johnson DB, Balko J, Wienert S, Bossuyt V, Michiels S, Ternes N, Burchardi N, Luen SJ, Savas P, Klauschen F, Watson PH, Nelson BH, Criscitiello C, O'Toole S, Larsimont D, de Wind R, Curigliano G, André F, Lacroix-Triki M, van de Vijver M, Rojo F, Floris G, Bedri S, Sparano J, Rimm D, Nielsen T, Kos Z, Hewitt S, Singh B, Farshid G, Loibl S, Allison KH, Tung N, Adams S, Willard-Gallo K, Horlings HM, Gandhi L, Moreira A, Hirsch F, Dieci MV, Urbanowicz M, Brcic I, Korski K, Gaire F, Koeppen H, Lo A, Giltnane J, Rebelatto MC, Steele KE, Zha J, Emancipator K, Juco JW, Denkert C, Reis-Filho J, Loi S, Fox SB. Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method from the International Immuno-Oncology Biomarkers Working Group: Part 2: TILs in Melanoma, Gastrointestinal Tract Carcinomas, Non-Small Cell Lung Carcinoma and Mesothelioma, Endometrial and Ovarian Carcinomas, Squamous Cell Carcinoma of the Head and Neck, Genitourinary Carcinomas, and Primary Brain Tumors. *Adv Anat Pathol* 2017; **24**: 311-335 [PMID: 28777143 DOI: 10.1097/PAP.0000000000000161]

12   **Bankhead P**, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, James JA, Salto-Tellez M, Hamilton PW. QuPath: Open source software for digital pathology image analysis. *Sci Rep* 2017; **7**: 16878 [PMID: 29203879 DOI: 10.1038/s41598-017-17204-5]

13   **Ono H**, Yao K, Fujishiro M, Oda I, Uedo N, Nimura S, Yahagi N, Iishi H, Oka M, Ajioka Y, Fujimoto K. Guidelines for endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer (second edition). *Dig Endosc* 2021; **33**: 4-20 [PMID: 33107115 DOI: 10.1111/den.13883]

14   **Li H**, Lu P, Lu Y, Liu CG, Xu HM, Wang SB, Chen JQ. Predictive factors for lymph node metastasis in poorly differentiated early gastric cancer and their impact on the surgical strategy. *World J Gastroenterol* 2008; **14**: 4222-4226 [PMID: 18636670 DOI: 10.3748/wjg.14.4222]

15   **Takizawa K**, Ono H, Hasuike N, Takashima A, Minashi K, Boku N, Kushima R, Katayama H, Ogawa G, Fukuda H.   A nonrandomized, single-arm confirmatory trial of expanded endoscopic submucosal dissection indication for undifferentiated early gastric cancer: Japan Clinical Oncology Group study (JCOG1009/1010). Gastric Cancer 2020: 1-13

16   **Oh SY**, Lee KG, Suh YS, Kim MA, Kong SH, Lee HJ, Kim WH, Yang HK. Lymph Node Metastasis in Mucosal Gastric Cancer: Reappraisal of Expanded Indication of Endoscopic Submucosal Dissection. *Ann Surg* 2017; **265**: 137-142 [PMID: 28009738 DOI: 10.1097/SLA.0000000000001649]

17   Pathologists CoA. Protocol for the Examination of Specimens from Patients with Carcinoma of the Stomach. College of American Pathologists: Northfield 2014

18   **Huh CW**, Jung DH, Kim JH, Lee YC, Kim H, Yoon SO, Youn YH, Park H, Lee SI, Choi SH, Cheong JH, Noh SH. Signet ring cell mixed histology may show more aggressive behavior than other histologies in early gastric cancer. *J Surg Oncol* 2013; **107**: 124-129 [PMID: 22991272 DOI: 10.1002/jso.23261]

19   **Seo HS**, Lee GE, Kang MG, Han KH, Jung ES, Song KY. Mixed Histology Is a Risk Factor for Lymph Node Metastasis in Early Gastric Cancer. *J Surg Res* 2019; **236**: 271-277 [PMID: 30694766 DOI: 10.1016/j.jss.2018.11.055]

20   **Quail DF**, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; **19**: 1423-1437 [PMID: 24202395 DOI: 10.1038/nm.3394]

21   **Ahn JY**, Choi KD, Choi JY, Kim MY, Lee JH, Choi KS, Kim DH, Song HJ, Lee GH, Jung HY, Kim JH. Procedure time of endoscopic submucosal dissection according to the size and location of early gastric cancers: analysis of 916 dissections performed by 4 experts. *Gastrointest Endosc* 2011; **73**: 911-916 [PMID: 21296348 DOI: 10.1016/j.gie.2010.11.046]

22   **Yoo JH**, Shin SJ, Lee KM, Choi JM, Wi JO, Kim DH, Lim SG, Hwang JC, Cheong JY, Yoo BM, Lee KJ, Kim JH, Cho SW. Risk factors for perforations associated with endoscopic submucosal dissection in gastric lesions: emphasis on perforation type. *Surg Endosc* 2012; **26**: 2456-2464 [PMID: 22398962 DOI: 10.1007/s00464-012-2211-x]

23   **Emura F**, Mejía J, Donneys A, Ricaurte O, Sabbagh L, Giraldo-Cadavid L, Oda I, Saito Y, Osorio C. Therapeutic outcomes of endoscopic submucosal dissection of differentiated early gastric cancer in a Western endoscopy setting (with video). *Gastrointest Endosc* 2015; **82**: 804-811 [PMID: 25952087 DOI: 10.1016/j.gie.2015.03.1960]

24   **Kim JH**, Nam HS, Choi CW, Kang DH, Kim HW, Park SB, Kim SJ, Hwang SH, Lee SH. Risk factors associated with difficult gastric endoscopic submucosal dissection: predicting difficult ESD. *Surg Endosc* 2017; **31**: 1617-1626 [PMID: 27495343 DOI: 10.1007/s00464-016-5149-6]

25   **Lee GH**, Park JW, Roh J, Kim YB, Lee E, Lim SG, Shin SJ, Lee KM, Noh CK. Association Between Waiting Time from Diagnosis to Endoscopic Submucosal Dissection and Non-curative Resection in Gastric Neoplasm. *Anticancer Res* 2021; **41**: 459-466 [PMID: 33419844 DOI: 10.21873/anticanres.14796]

26   **Lehnert T**, Erlandson RA, Decosse JJ. Lymph and blood capillaries of the human gastric mucosa. A morphologic basis for metastasis in early gastric carcinoma. *Gastroenterology* 1985; **89**: 939-950 [PMID: 4043674 DOI: 10.1016/0016-5085(85)90192-1]

27   **Chandler C**, Liu T, Buckanovich R, Coffman LG. The double edge sword of fibrosis in cancer. *Transl Res* 2019; **209**: 55-67 [PMID: 30871956 DOI: 10.1016/j.trsl.2019.02.006]

28   **Song SY**, Park S, Kim S, Son HJ, Rhee JC. Characteristics of intramucosal gastric carcinoma with lymph node metastatic disease. *Histopathology* 2004; **44**: 437-444 [PMID: 15139991 DOI: 10.1111/j.1365-2559.2004.01870.x]

29   **Friedl P**, Locker J, Sahai E, Segall JE. Classifying collective cancer cell invasion. *Nat Cell Biol* 2012; **14**: 777-783 [PMID: 22854810 DOI: 10.1038/ncb2548]

30   **Chen HC**, Chu RY, Hsu PN, Hsu PI, Lu JY, Lai KH, Tseng HH, Chou NH, Huang MS, Tseng CJ, Hsiao M. Loss of E-cadherin expression correlates with poor differentiation and invasion into adjacent organs in gastric adenocarcinomas. *Cancer Lett* 2003; **201**: 97-106 [PMID: 14580691 DOI: 10.1016/j.canlet.2003.07.007]

Retrospective Cohort Study

Validation model of fibrosis-8 index score to predict significant fibrosis among patients with nonalcoholic fatty liver disease

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Abstract

BACKGROUND

Identifying hepatic fibrosis is crucial for nonalcoholic fatty liver disease (NAFLD)

management. The fibrosis-8 (FIB-8) score, recently developed by incorporating four additional variables into the fibrosis-4 (FIB-4) score, showed better performance in predicting significant fibrosis in NAFLD.

AIM
To validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 and FIB-4 scores and NAFLD fibrosis score (NFS) for predicting significant fibrosis.

METHODS
We collected the data of biopsy-proven NAFLD patients from three Asian centers in three countries. All the patients with available variables for the FIB-4 score (age, platelet count, and aspartate and alanine aminotransferase levels) and FIB-8 score (the FIB-4 variables plus 4 additional parameters: The body mass index (BMI), albumin to globulin ratio, gamma-glutamyl transferase level, and presence of diabetes mellitus) were included. The fibrosis stage was scored using nonalcoholic steatohepatitis CRN criteria, and significant fibrosis was defined as at least fibrosis stage 2.

RESULTS
A total of 511 patients with biopsy-proven NAFLD and complete data were included for validation. Of these 511 patients, 271 (53.0%) were female, with a median age of 51 (interquartile range: 41, 58) years. The median BMI was 29 (26.3, 32.6) kg/m^2, and 268 (52.4%) had diabetes. Among the 511 NAFLD patients, 157 (30.7%) had significant fibrosis (≥ F2). The areas under the receiver operating characteristic curves of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively. The FIB-8 score demonstrated significantly better performance for predicting significant fibrosis than the NFS (P = 0.001) and was also clinically superior to FIB-4, although statistical significance was not reached (P = 0.073). The low cutoff point of the FIB-8 score for predicting significant fibrosis of 0.88 showed 92.36% sensitivity, and the high cutoff point of the FIB-8 score for predicting significant fibrosis of 1.77 showed 67.51% specificity.

CONCLUSION
We demonstrated that the FIB-8 score had significantly better performance for predicting significant fibrosis in NAFLD patients than the NFS, as well as clinically superior performance *vs* the FIB-4 score in an Asian population. A novel simple fibrosis score comprising commonly accessible basic laboratories may be beneficial to use for an initial assessment in primary care units, excluding patients with significant liver fibrosis and aiding in patient selection for further hepatologist referral.

Core Tip: Noninvasive diagnosis of hepatic fibrosis is crucial for nonalcoholic fatty liver disease (NAFLD). The fibrosis-8 (FIB-8) score was recently developed by incorporating four additional variables into the fibrosis-4 (FIB-4) score. The diagnostic performance of the FIB-8 score exhibited higher accuracy in diagnosing significant fibrosis (≥ F2) than the NAFLD fibrosis score but was not superior to the FIB-4 score in our Asian cohort population. We postulated that gamma-glutamyl transferase might be an additional variable that predicts significant fibrosis in NAFLD patients.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a global health issue and has become the most common liver disease in Western countries, accounting for an estimated 25% of the adult population[1] and affecting an estimated 25%–30% of the adult population in the Asia Pacific region[2]. A meta-analysis in Asia during 1999 to 2019, described the overall pooled incidence rate was 50.9 *per* 1000 person-years[3]. According to our previous study, the prevalence of significant fibrosis (defined as ≥ F2 fibrosis) is 18.4% in asymptomatic NAFLD patients[4]. Nonalcoholic steatohepatitis (NASH) has emerged as the most common cause of cryptogenic cirrhosis and hepatocellular carcinoma worldwide. The presence of hepatic fibrosis is the major determinant of future risk of mortality and liver-related morbidity[5], and detecting significant fibrosis is crucial for NAFLD because no well-accepted and proven therapy is available for this disease to date[6]. However, patients with F2 or higher are at a higher risk of long-term liver-related death than patients with F0-1. Those with significant fibrosis should be intensively followed up or considered to participate in the therapeutic trial for NAFLD.

Liver biopsy remains the gold standard for evaluating hepatic fibrosis. However, because of several drawbacks, including invasiveness, the risk of bleeding complications, intrinsic sampling and pathologist reader variability[7], and cost, noninvasive tests are more practical. Thus, the 2018 American Association for the Study of Liver Diseases (AASLD) practice guidance recommends the use of the fibrosis-4 (FIB-4) score, the NAFLD fibrosis score (NFS), vibration-controlled transient elastography, and magnetic resonance elastography[8] to identify those at low or high risk for advanced fibrosis [bridging fibrosis (F3) or cirrhosis (F4)]. Noninvasive tests using only clinical and routine laboratory parameters are inexpensive and particularly important in primary care or resource-limited settings where the pretest probability of advanced fibrosis is low because these scores have good negative predictive values (NPVs) to exclude advanced fibrosis[9]. Therefore, using simple fibrosis scores as an initial assessment in primary care is reasonable. The FIB-4 score comprises four parameters, age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelets, while the NFS score comprises six parameters in addition to those comprising the FIB-4 score, such as the body mass index (BMI), presence of diabetes, and serum albumin level[10].

According to Sripongpun *et al*[11], their AASLD 2019 abstract reported a new model for a fibrosis-8 score (FIB-8) score developed by incorporating the following four additional variables: BMI, albumin/globulin (A/G) ratio, gamma-glutamyl transferase (GGT) level, and diabetes. The subjects were enrolled in the PIVENS and FLINT trials, of which 522 participants all had histologically confirmed NASH[12,13]. The optimal low and high cutoffs for the FIB-8 score to exclude and include F ≥ 2 were < 0.88 and ≥ 1.77, respectively, with a sensitivity of 95.3% and a specificity of 79.2%. The areas under the receiver operating characteristic curves (AUROCs) of the FIB-8 score were 0.79 and 0.78 in the training and validation datasets, respectively. The FIB-8 score provided significantly better AUROCs than the FIB-4 score ($P < 0.001$) and NFS ($P = 0.005$) in the validation dataset for predicting significant and advanced fibrosis in NAFLD patients. Following the study, the field test and validation of the FIB-8 score in a real-world cohort of NAFLD patients revealed that the AUROCs of the FIB-8 score were 0.84 with imputed data ($n = 130$) and 0.91 when only patients with complete data without imputation were included ($n = 31$). The FIB-8 score again outperformed the FIB-4 score and NFS, with AUROCs of 0.86 *vs* 0.80 and 0.77, respectively, for diagnosing advanced fibrosis (F3)[14].

To our best knowledge, no validation of the FIB-8 score has been reported in a larger cohort. Therefore, this study was to validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis.

MATERIALS AND METHODS

Study population and data collection

We collected the data of biopsy-proven NAFLD patients from the following three Asian centers in three countries: (1) Chulalongkorn University, Thailand; (2) The Chinese University of Hong Kong, Hong Kong; and (3) University of Malaya, Malaysia. The data from Thailand were collected from April 2008 to May 2019, those from Hong Kong were collected from July 2006 to November 2017, and those from Malaysia were collected from November 2012 to October 2015.

NAFLD was diagnosed based on ultrasonographic findings of fatty liver as well as transient elastography and the exclusion of viral hepatitis B and C infection, significant alcohol intake, and current usage of medications causing hepatic steatosis. Only patients with biopsy-proven NAFLD were included. Patients with other causes of chronic liver disease, incomplete histological data, and without significant hepatic steatosis were excluded. The laboratory data for the FIB-4 score (age, platelet count, and aspartate and ALT levels), FIB-8 score [the FIB-4 variables plus 4 additional parameters: The BMI, albumin to globulin ratio, gamma-glutamyl transferase level, and presence of diabetes mellitus (DM)], and the NFS were collected. The time interval between the enrolled laboratories and the date of liver biopsy was within 1 year. The fibrosis stage was scored using the NASH Clinical Research Network (CRN) criteria, and significant fibrosis was defined as at least fibrosis stage 2 (F ≥ 2).

Noninvasive methods

We validated the noninvasive methods from the FIB-8 score, FIB-4 score, and NFS and the test variables for predicting significant fibrosis (Table 1)[11,15,16].

Outcomes

We aimed to validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2) in an Asian cohort.

Ethical permission

The study was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB number 238/59). This is a retrospective study, and signed informed consent was waived by the Ethics Committee. The analysis used anonymous clinical data after each patient agreed to treatment by written consent.

Statistical analysis

Categorical and continuous variables were compared between patients with and without significant fibrosis using Chi-squared and Student's t-test or the Wilcoxon rank-sum test (according to the distribution of the data), respectively. Most of the numerical values did not follow a normal distribution and were expressed as medians and interquartile ranges. The diagnostic performance of each scoring system was then evaluated using receiver operating characteristic curves, and comparisons between the correlated AUROCs were performed using DeLong's test[17]. The sensitivities (Sens) and specificities (Spec) of each scoring system were analyzed using the given low and high cutoffs for predicting F2, as reported previously-*i.e.*, 0.88 and 1.77 for the FIB-8 score, 0.81 and 1.81 for the FIB-4 score, and -2.45 and 0.03 for the NFS, respectively[11,18]. All statistical analyses were performed using the SPSS statistical analysis package (version 18.0.0; SPSS Inc., Chicago, Illinois, United States), Stata (version 15; StataCorp), and R program version 4.1.1. A *P* value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

A total of 1013 patients with biopsy-proven NAFLD were included in the database. Of those, 511 patients had complete data on variables, including the NFS and FIB-4 and FIB-8 scores, and were eligible for the current study (Figure 1). Of the 511 patients, 271 (53.0%) were female, with a median age of 51 [interquartile range (IQR): 41, 58] years. The median BMI was 29 (26.3, 32.6) kg/m², and 268 (52.4%) had diabetes. Among the 511 NAFLD patients, 157 (30.7%), 88 (17.2%), and 16 (3.1%) patients had significant fibrosis (≥ F2), advanced fibrosis (≥ F3), and cirrhosis (F4), respectively. The baseline characteristics comparing NAFLD F0–1 and significant fibrosis (F ≥ 2) are shown in Table 2. The significant factors associated with significant fibrosis were an older age [55 (48, 61) *vs* 49.5 (39, 57) years; *P* < 0.001], the presence of diabetes (71.3% *vs* 44.0%; *P* < 0.001), higher levels of AST [53.5 (36, 75) *vs* 35 (26, 52) U/L; *P* < 0.001], ALT [75 (50, 111) *vs* 59.5 (40, 98) U/L; *P* < 0.001] and GGT [81 (48, 151) *vs* 56.5 (35, 92) U/L; *P* < 0.001], a lower platelet count [230 (189, 277) *vs* 266 (226.8, 302) × 10⁹/cu.mm; *P* < 0.001], lower levels of total cholesterol [182 (159, 209) *vs* 193 (170, 220) mg/dL; *P* = 0.004] and LDL-cholesterol [107 (85, 132) *vs* 116 (96, 143) mg/dL; *P* = 0.003], and a higher median Controlled Attenuation Parameter (CAP) [324 (294, 347) *vs* 299 (211, 339) dB/m] (Table 2).

Performance of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2)

The AUROCs of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis were 0.774 (95%CI: 0.729-0.820), 0.743 (95%CI: 0.695-0.791), and 0.680 (95%CI: 0.630-0.730), respectively (Figure 2). The FIB-8 score showed a significantly better performance for predicting significant fibrosis (≥ F2) than the NFS (*P* = 0.001) and was numerically higher than the FIB-4 score, but the difference was not statistically significant (*P* = 0.073). The sensitivities and specificities of the cutoffs specified to exclude and include significant fibrosis for each score are reported in Table 3.

Diagnostic accuracy of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2) by age group

The cohort was stratified by age into three groups: Age < 35 (*n* = 66), 35-65 (*n* = 412), and > 65 years (*n* = 33). The AUROCs of the FIB-8 score, FIB-4 score, and NFS in patients aged 35-65 years for predicting significant fibrosis were 0.79, 0.76, and 0.68, respectively. This patient group comprised most of the cohort and had similar diagnostic performance results as the entire cohort. However, the FIB-8 score, FIB-4 score, and NFS were poor in patients aged < 35 years (AUROC: 0.55, 0.59, and 0.70, respectively) and > 65 years (AUROC: 0.66, 0.71, and 0.54, respectively). The number of patients in each age group

Table 1 Details of the three noninvasive methods used in this study

Index	Number of parameters and variables	Formula	Ref.
FIB-8 index	8; age, AST, ALT, platelets, BMI, albumin/globulin, GGT, diabetes	FIB4 + 0.025 × BMI (kg/m^2) - 0.702 × (albumin/globulin ratio) + 0.004 × GGT (U/L) + 0.858 × diabetes (yes = 1, no = 0)[1]	Sripongpun *et al*[11], 2019
FIB-4 index	4; age, AST, ALT, platelets	Age (years) × AST (U/L)/[platelet count (10^9/L) × √ALT (U/L)]	Sterling *et al*[15], 2006
NFS	6; age, BMI, diabetes, AST/ALT, platelets, albumin	-1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × impaired fasting glucose/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio - 0.013 × platelet count (× 10^9/L) - 0.66 × albumin (g/dL)	Angulo *et al*[16], 2007

[1]The FIB-8 score in the abstract was calculated by 1.3 × FIB-4 + 0.03 × BMI - 0.93 × (albumin/globulin ratio) + 0.005 × GGT (U/L) + 1.1 × diabetes (yes = 1, no = 0), which is slightly different from the actual formula presented at the liver meeting congress (the score in the table). We have contacted the authors of the abstract and were informed that the actual formula to use is the one shown in the table.

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves; GGT: Gamma-glutamyl transferase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index.

and center is shown in Supplementary Table 1. A detailed summary of the AUROC, sensitivity, specificity, positive predictive value, and NPV for the FIB-8 score, FIB-4 score, and the NFS is shown in Supplementary Table 2.

DISCUSSION

Based on the results of the present study, we validated the diagnostic performance of the FIB-8 score, FIB-4 score, and NFS score in 511 biopsy-proven NAFLD patients for predicting significant fibrosis. The main issue affecting the diagnostic ability of new methods for detecting liver fibrosis in NAFLD patients is the prevalence of fibrosis among the particular population. Our results demonstrated that the overall prevalence rates of significant fibrosis (≥ F2), advanced fibrosis (≥ F3), and cirrhosis (F4) were 157 (30.7%), 88 (17.2%), and 16 (3.1%), respectively. The mean incidence rates of significant fibrosis from previous publications were 52.5% and 35.4% in the PIVENS plus FLINT trials and a Stanford University trial, respectively[11,14] (Table 4). The remarkable aspects were as follows: (1) Our study had a lower incidence of fibrosis than the first cohort; (2) Among the noninvasive methods, the FIB-8 score and NFS included the BMI in their models, and our cohort had a lower mean BMI than previous reports (30.4 kg/m^2 *vs* 34.0 and 31.5 kg/m^2), which might have resulted in lower percentages of sensitivity and specificity in our cohort than those previously reported; and (3) GGT is a uniquely incorporated variable in the new FIB-8 scoring system. Some reported studies have demonstrated that a higher GGT level is a risk factor for advanced fibrosis in NAFLD[19,20]. Additionally, considering NAFLD patients with type 2 DM, a serum GGT level over 82 U/L was independently associated with advanced fibrosis using noninvasive methods in multivariate analysis ($P = 0.004$)[21]. In our study, the baseline characteristics correlatively showed that a higher level of median GGT was a significant factor associated with significant fibrosis [81 (IQR: 48, 151) *vs* 56.5 (35, 92); $P < 0.001$]. We postulated that GGT may be an additional variable predicting significant fibrosis in NAFLD patients. The diagnostic performance of the FIB-8 score exhibited higher accuracy for diagnosing significant fibrosis (≥ F2) than the NFS but was not superior to the FIB-4 score in previous studies or our study; the AUROCs for the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively (FIB-8 *vs* NFS, $P = 0.001$; FIB-8 *vs* FIB-4, $P = 0.073$). The sensitivities of the low cutoff of FIB-8 score to exclude significant fibrosis was 92.36%. Consequently, the high sensitivity and NPV for excluded significant fibrosis may be beneficial in primary care units and to select patients for further hepatologist referral. However, the limited specificity of the high cutoff of FIB-8 score to include significant fibrosis may require further step assessment instance transient elastography.

Furthermore, our results demonstrated that the FIB-4 score offered better diagnostic performance than the NFS score ($P < 0.001$). According to meta-analysis results from Castera[10], the FIB-4 score and NFS showed the best diagnostic performance for detecting advanced fibrosis compared with other blood-based models. However, this meta-analysis included studies that used different cut-off thresholds. Furthermore, a recent meta-analysis from Castellana *et al*[22] reported a head-to-head comparison of the FIB-4 score and NFS from 18 studies that used consistent cutoffs. The FIB-4 score offered higher performance for including and NFS for excluding advanced fibrosis. However, our studies used different cutoffs and aimed to predict significant fibrosis, not advanced fibrosis. Consequently, our cohort was not suitable to compare the FIB-4 score and NFS.

Table 2 Characteristics of patients with F0-1 fibrosis compared to those with F ≥ 2 fibrosis stage (*n* = 511)

Variables	Total (*n* = 511)	Fibrosis stage F0-1 (*n* = 354)	Fibrosis stage ≥ F2 (*n* = 157)	*P* value
Age (yr), median (IQR)	51 (41, 58)	49.5 (39, 57)	55 (48, 61)	< 0.001
Sex				0.138
Male, *n* (%)	240 (47.0)	174 (49.2)	66 (42.0)	
Female, *n* (%)	271 (53.0)	180 (50.8)	91 (58.0)	
BMI (kg/m^2), median (IQR)	29.0 (26.3, 32.6)	28.8 (26.2, 31.9)	29.5 (26.3, 33.8)	0.099
Diabetes, *n* (%)	268 (52.4)	156 (44.0)	112 (71.3)	< 0.001
Albumin (g/dL), median (IQR)	4.4 (4.1, 4.6)	4.4 (4.2, 4.6)	4.30 (4.0, 4.6)	0.053
Globulin (g/dL), median (IQR)	3.4 (3.0, 3.8)	3.4 (3.0, 3.8)	3.5 (3.1, 3.8)	0.21
AST (U/L), median (IQR)	39 (28, 60)	35 (26, 52)	53.5 (36, 75)	<0.001
ALT (U/L), median (IQR)	65 (42, 101)	59.5 (40, 98)	75 (50, 111)	< 0.001
GGT (U/L), median (IQR)	63 (37, 108)	56.5 (35, 92)	81 (48, 151)	< 0.001
Platelet (× 10^9/μL), median (IQR)	254 (213, 297)	266 (226.8, 302)	230 (189, 277)	< 0.001
Hemoglobin (g/dL), median (IQR)	14.2 (13.3, 15.2)	14.2 (13.4, 15.2)	14.1 (13.3, 15.2)	0.393
White blood cells (cells/μL), median (IQR)	7430 (6060, 8700)	7400 (6100, 8725)	7500 (5950, 8695)	0.768
INR, median (IQR)	1.01 (0.96, 1.06)	1.00 (0.95, 1.07)	1.01 (0.97, 10.6)	0.625
Total cholesterol (mg/dL), median (IQR)	189.5 (166, 217)	193 (170, 220)	182 (159, 209)	0.004
LDL-cholesterol (mg/dL), median (IQR)	115 (92, 139)	116 (96, 143)	107 (85, 132)	0.003
HDL-cholesterol (mg/dL), median (IQR)	46 (39, 52)	46 (39, 53)	44 (38, 50)	0.168
Triglyceride (mg/dL), median (IQR)	120 (77, 157)	120 (77, 155)	119 (80, 159)	0.483
HbA1C (%), median (IQR)	6.1 (5.6, 7.2)	5.9 (5.5, 6.8)	6.8 (5.8, 7.6)	< 0.001
Fibrosis stage, *n* (%)				< 0.001
0	151 (29.5)	151 (42.7)	0 (0)	
1	203 (39.7)	203 (57.3)	0 (0)	
2	69 (13.5)	0 (0)	69 (43.9)	
3	72 (14.1)	0 (0)	72 (45.9)	
4	16 (3.1)	0 (0)	16 (10.2)	
Median CAP (dB/m), median (IQR)	308.5 (230, 342)	299 (211, 339)	324 (294, 347)	< 0.001
Median TE (kPa), median (IQR)	7.6 (5.6, 10.9)	6.6 (5.1, 8.8)	11.1 (8.6, 15.5)	< 0.001
FIB-8, median (IQR)	2.0 (1.2, 2.9)	1.8 (1.1, 2.4)	3.0 (2.2, 4.0)	< 0.001
FIB-4, median (IQR)	1.0 (0.7, 1.5)	0.8 (0.6, 1.2)	1.5 (1.0, 2.1)	< 0.001
NFS, mean ± SD	-1.8 ± 1.5	-2.0 ± 1.4	-1.2 ± 1.3	< 0.001

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves; GGT: Gamma-glutamyl transferase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index; CAP: Controlled Attenuation Parameter.

Additionally, our results demonstrated the performance of the FIB-8 score, FIB-4 score, and NFS in patients aged > 65 years (AUROC: 0.66, 0.71, and 0.54, respectively). The performance was poor in patients aged < 35 years (AUROC: 0.55, 0.59, and 0.70, respectively). Thus, these scores have insufficient accuracy for use in NAFLD patients in extreme age groups. Similarly, McPherson *et al*[23] demonstrated age as a confounding factor for the accurate noninvasive scoring system predicting advanced fibrosis [23]. The FIB-8 score has low accuracy for predicting significant fibrosis in NAFLD patients, similar to

Table 3 Performance of fibrosis-8, fibrosis-4, and nonalcoholic fatty liver disease fibrosis score for predicting significant fibrosis (F ≥ 2) in the Asian population (*n* = 511)

	FIB-8 score	FIB-4 score	NFS
AUC for predicting ≥ F2 fibrosis	0.77[a,b]	0.74	0.68
95% confidence interval	0.73-0.82	0.70-0.79	0.63-0.73
Low and high cutoffs for ≥ F2 fibrosis	0.88 and 1.77	0.81 and 1.81 (17)	-2.45 and 0.03 (17)
Sensitivity (according to the low cutoff)	92.36%	80.25%	80.89%
Specificity (according to the high cutoff)	67.51%	93.50%	93.20%
Proportion of patients in low/indeterminate/high group	18.8/35.4/45.8%	38.2/47.1/14.7%	31.9/58.1/10%

[a]P = 0.001 compared with NFS.

[b]P = 0.073 compared with FIB-4.

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves.

Table 4 Comparison of study population using the fibrosis-8 score for predicting significant fibrosis (F ≥ 2)

Variable	Data from AASLD 2019, *n* = 522	FIB-8 score validation (EASL 2020), *n* = 130	FIB-8 score validation (Our cohort), *n* = 511
Population	Mean age: 49 ± 12. Female: 62.5%; BMI: 34 ± 7 kg/m^2; DM: 30%; ≥ F2: 52.5%	Mean age: 52.4; Female: 53.1%; BMI: 31.5 kg/m^2; DM: 34%; ≥ F2: 35.4%	Mean age: 49.3 ± 11.9; Female: 53.0%; BMI: 30.4 ± 7.1 kg/m^2; DM: 52.4%; ≥ F2: 30.7%
Sensitivity and specificity %	86.7% and 82.7%, respectively, Validation set	> 90%, 80.6%	92.3%, 67.5%
AUC for predicting > F2 fibrosis	0.78, Validation set	0.84	0.77
Performance superior to	> FIB-4 score; P < 0.001; > NFS; P = 0.005	> FIB-4 score (AUC 0.80); > NFS (AUC 0.77)	> FIB-4 score; P = 0.073; > NFS; P = 0.001

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver; AUROC: Areas under the receiver operating characteristic curves; BMI: Body mass index; DM: diabetes mellitus.
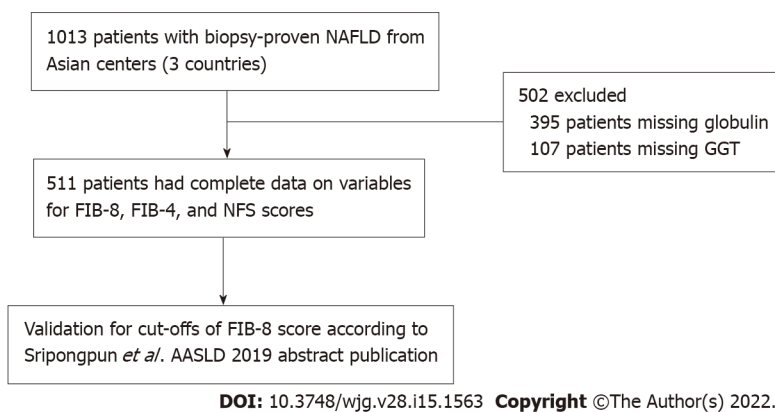
Figure 1 Flow diagram of the study population. NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AASLD: American Association for the Study of Liver Diseases; GGT: Gamma-glutamyl transferase.

the FIB-4 score and NFS in patients aged < 35 and > 65 years.

Our study had limitations. First, we had limited complete data for half of our database because of the lack of either globulin or GGT. In usual clinical practice, clinicians do not routinely check both laboratory parameters, and no added value exists for observing or monitoring these values in patients. The second limitation of our study was the lower incidence of fibrosis in our cohort *vs* other cohorts. The differences in fibrosis may have diagnostic value for novel fibrosis scores for validation. Validations

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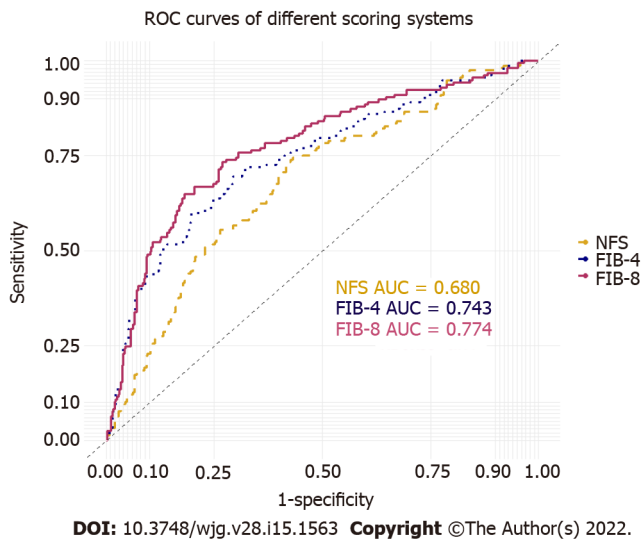
Figure 2 Receiver operating characteristic curves of the fibrosis-8 score, fibrosis-4 score, and nonalcoholic fatty liver disease fibrosis score for predicting significant fibrosis (F ≥ 2) in the Asian population (*n* = 511). NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves.

in larger cohorts are needed.

To our best knowledge, our study is the first to report a new validation model of the FIB-8 score for predicting significant fibrosis among patients with NAFLD in an Asian population. The FIB-8 score yielded higher accuracy in diagnosing significant fibrosis than the NFS. Additionally, the FIB-8 score was non-inferior but insignificantly superior to the FIB-4 score. A novel simple fibrosis score comprising commonly accessible basic laboratories may be additionally used to add previous fibrosis scores for an initial assessment in primary care units and to select patients for further hepatologist referral.

CONCLUSION

The new, simple fibrosis FIB-8 score had significantly better performance for predicting significant fibrosis in NAFLD patients than the NFS and was non-inferior but insignificantly superior to the FIB-4 score in the Asian population. A simple fibrosis score comprising commonly accessible basic laboratories may be used for an initial assessment in primary care units and to select patients for further hepatologist referral.

ARTICLE HIGHLIGHTS

Research background

In the nonalcoholic fatty liver disease (NAFLD) population, noninvasive fibrosis scores, such as the fibrosis-4 (FIB-4) score and NAFLD fibrosis score (NFS), are generally applied in clinical practice guidelines. The novel fibrosis-8 (FIB-8) score yielded higher accuracy in diagnosing significant fibrosis in a previously reported cohort. A larger cohort may provide more reliability and benefit in clinical practice.

Research motivation

A noninvasive fibrosis score in NAFLD patients using only routine laboratory parameters is particularly important in initial assessment in the primary care unit or resource-limited conditions. We proposed the novel FIB-8 score, which incorporates the additional variables body mass index (BMI), the A/G ratio, gamma-glutamyl transferase (GGT), and diabetes into the FIB-4 score. The additional variables, particularly GGT, may provide better diagnostic accuracy for predicting significant fibrosis in NAFLD patients.

Research objectives

We aimed to validate the FIB-8 score among patients with a biopsy-proven NAFLD cohort and to compare the diagnostic performance of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis.

Research methods

This was a retrospective study involving 1013 biopsy-proven NAFLD patients from 3 Asian centers in 3 countries in an Asian population. All the patients with available baseline biochemical tests for the FIB-8 score calculation and all related variables for predicting liver fibrosis were included.

Research results

A total of 1013 patients were included in the final analysis. Of those, 511 patients had complete data on the variables, including the NFS and FIB-4 and FIB-8 scores. One hundred fifty-seven (30.7%) patients had significant fibrosis (≥ F2). The areas under the receiver operating characteristic curves of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively. The FIB-8 score had significantly better performance for predicting significant fibrosis than the NFS (P = 0.001) but was not superior to the FIB-4 score (P = 0.073). The low cutoff point of the FIB-8 score for predicting significant fibrosis of 0.88 showed 92.36% sensitivity, and the high cutoff point of the FIB-8 score for predicting significant fibrosis of 1.77 had 67.51% specificity.

Research conclusions

The FIB-8 score, which incorporates the additional variables of the BMI, A/G ratio, GGT level, and diabetes into the FIB-4 score, yielded better performance for predicting significant fibrosis in NAFLD patients than the NFS but was not superior to the FIB-4 score in the Asian population. A simple fibrosis score comprising commonly accessible basic laboratories may be used for an initial assessment in primary care units.

Research perspectives

Future prospective studies are needed to compare the diagnostic accuracy of various noninvasive scores for predicting significant fibrosis and staging fibrosis.

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FOOTNOTES

Author contributions: Treeprasertsuk S designed the study; Pitisuttithum P, Chan WK, Wong VWS, and Treeprasertsuk S contributed to data acquisition; Mahadeva S and Wong GLH recruited and managed the patients; Mustapha NRN and Leung HHW performed the histological assessment; Prasoppokakorn T, Pitisuttithum P, Sripongpun P, and Treeprasertsuk S analyzed and interpreted the data; Prasoppokakorn T drafted the manuscript; Chan WK, Sripongpun P, Wong VWS, and Treeprasertsuk S revised the manuscript critically for important intellectual content; all the authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, No. 238/59.

Informed consent statement: This is a retrospective study, and an exemption of a signed informed consent application was approved by the Ethics Committee. The analysis used anonymous clinical data after each patient agreed to treatment by written consent.

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

Data sharing statement: No additional data are available.

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REFERENCES

1   **Younossi ZM**. Non-alcoholic fatty liver disease - A global public health perspective. *J Hepatol* 2019; **70**: 531-544 [PMID: 30414863 DOI: 10.1016/j.jhep.2018.10.033]

2   **Chan WK**, Treeprasertsuk S, Imajo K, Nakajima A, Seki Y, Kasama K, Kakizaki S, Fan JG, Song MJ, Yoon SK, Dan YY, Lesmana L, Ho KY, Goh KL, Wong VW. Clinical features and treatment of nonalcoholic fatty liver disease across the Asia Pacific region-the GO ASIA initiative. *Aliment Pharmacol Ther* 2018; **47**: 816-825 [PMID: 29333610 DOI: 10.1111/apt.14506]

3   **Li J**, Zou B, Yeo YH, Feng Y, Xie X, Lee DH, Fujii H, Wu Y, Kam LY, Ji F, Li X, Chien N, Wei M, Ogawa E, Zhao C, Wu X, Stave CD, Henry L, Barnett S, Takahashi H, Furusyo N, Eguchi Y, Hsu YC, Lee TY, Ren W, Qin C, Jun DW, Toyoda H, Wong VW, Cheung R, Zhu Q, Nguyen MH. Prevalence, incidence, and outcome of non-alcoholic fatty liver disease in Asia, 1999-2019: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2019; **4**: 389-398 [PMID: 30902670 DOI: 10.1016/S2468-1253(19)30039-1]

4   **Teeratorn N**, Piyachaturawat P, Thanapirom K, Chaiteerakij R, Sonsiri K, Komolmit P, Tangkijvanich P, Rerknimitr R, Adams L, Treeprasertsuk S. Screening for non-alcoholic fatty liver disease in community setting: A cohort study using controlled attenuation parameter-transient elastography. *JGH Open* 2020; **4**: 245-250 [PMID: 32280772 DOI: 10.1002/jgh3.12252]

5   **Taylor RS**, Taylor RJ, Bayliss S, Hagström H, Nasr P, Schattenberg JM, Ishigami M, Toyoda H, Wai-Sun Wong V, Peleg N, Shlomai A, Sebastiani G, Seko Y, Bhala N, Younossi ZM, Anstee QM, McPherson S, Newsome PN. Association Between Fibrosis Stage and Outcomes of Patients With Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis. *Gastroenterology* 2020; **158**: 1611-1625.e12 [PMID: 32027911 DOI: 10.1053/j.gastro.2020.01.043]

6   **Tarantino G**, Citro V, Capone D. Nonalcoholic Fatty Liver Disease: A Challenge from Mechanisms to Therapy. *J Clin Med* 2019; **9** [PMID: 31861591 DOI: 10.3390/jcm9010015]

7   **Davison BA**, Harrison SA, Cotter G, Alkhouri N, Sanyal A, Edwards C, Colca JR, Iwashita J, Koch GG, Dittrich HC. Suboptimal reliability of liver biopsy evaluation has implications for randomized clinical trials. *J Hepatol* 2020; **73**: 1322-1332 [PMID: 32610115 DOI: 10.1016/j.jhep.2020.06.025]

8   **Chalasani N**, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, Brunt EM, Sanyal AJ. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**: 328-357 [PMID: 28714183 DOI: 10.1002/hep.29367]

9   **Mahady SE**, Macaskill P, Craig JC, Wong GLH, Chu WCW, Chan HLY, George J, Wong VWS. Diagnostic Accuracy of Noninvasive Fibrosis Scores in a Population of Individuals With a Low Prevalence of Fibrosis. *Clin Gastroenterol Hepatol* 2017; **15**: 1453-1460.e1 [PMID: 28286195 DOI: 10.1016/j.cgh.2017.02.031]

10   **Castera L**. Non-invasive tests for liver fibrosis in NAFLD: Creating pathways between primary healthcare and liver clinics. *Liver Int* 2020; **40** Suppl 1: 77-81 [PMID: 32077617 DOI: 10.1111/liv.14347]

11   **Sripongpun P**, Ajitha Mannalithara DK, Alexis Touros and W. Ray Kim. FIB-8 score: A model incorporating additional common variables into the FIB-4 score affords better prediction of significant fibrosis in non-alcoholic fatty liver disease. *AASLD Abstract publication* 2019; 1723

12   **Chalasani NP**, Sanyal AJ, Kowdley KV, Robuck PR, Hoofnagle J, Kleiner DE, Unalp A, Tonascia J; NASH CRN Research Group. Pioglitazone versus vitamin E versus placebo for the treatment of non-diabetic patients with non-alcoholic steatohepatitis: PIVENS trial design. *Contemp Clin Trials* 2009; **30**: 88-96 [PMID: 18804555 DOI: 10.1016/j.cct.2008.09.003]

13   **Neuschwander-Tetri BA**, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E; NASH Clinical Research Network. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015; **385**: 956-965 [PMID: 25468160 DOI: 10.1016/S0140-6736(14)61933-4]

14   **Sripongpun P**, Donghee Kim AM, W. Ray Kim. The FIB-8 score: validation of a model to screen patients with non-alcoholic fatty liver disease for significant fibrosis. *EASL The Digital Inter Liver Con 2020* 2020; FRI072

15   **Sterling RK**, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, S Sulkowski M, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317-1325 [PMID: 16729309 DOI: 10.1002/hep.21178]

16   **Angulo P**, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846-854 [PMID: 17393509 DOI:

10.1002/hep.21496]

17   **DeLong ER**, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988; **44**: 837-845 [PMID: 3203132]

18   **Siddiqui MS**, Yamada G, Vuppalanchi R, Van Natta M, Loomba R, Guy C, Brandman D, Tonascia J, Chalasani N, Neuschwander-Tetri B, Sanyal AJ; NASH Clinical Research Network. Diagnostic Accuracy of Noninvasive Fibrosis Models to Detect Change in Fibrosis Stage. *Clin Gastroenterol Hepatol* 2019; **17**: 1877-1885.e5 [PMID: 30616027 DOI: 10.1016/j.cgh.2018.12.031]

19   **Tahan V**, Canbakan B, Balci H, Dane F, Akin H, Can G, Hatemi I, Olgac V, Sonsuz A, Ozbay G, Yurdakul I, Senturk H. Serum gamma-glutamyltranspeptidase distinguishes non-alcoholic fatty liver disease at high risk. *Hepatogastroenterology* 2008; **55**: 1433-1438 [PMID: 18795706]

20   **Fujii H**, Doi H, Ko T, Fukuma T, Kadono T, Asaeda K, Kobayashi R, Nakano T, Doi T, Nakatsugawa Y, Yamada S, Nishimura T, Tomatsuri N, Sato H, Okuyama Y, Kimura H, Kishimoto E, Nakabe N, Shima T. Frequently abnormal serum gamma-glutamyl transferase activity is associated with future development of fatty liver: a retrospective cohort study. *BMC Gastroenterol* 2020; **20**: 217 [PMID: 32650722 DOI: 10.1186/s12876-020-01369-x]

21   **Arab JP**, Barrera F, Gallego C, Valderas JP, Uribe S, Tejos C, Serrano C, Huete Á, Liberona J, Labbé P, Quiroga T, Benítez C, Irarrázaval P, Riquelme A, Arrese M. High prevalence of undiagnosed liver cirrhosis and advanced fibrosis in type 2 diabetic patients. *Ann Hepatol* 2016; **15**: 721-728 [PMID: 27493111 DOI: 10.5604/16652681.1212434]

22   **Castellana M**, Donghia R, Guerra V, Procino F, Castellana F, Zupo R, Lampignano L, Sardone R, De Pergola G, Romanelli F, Trimboli P, Giannelli G. Fibrosis-4 Index vs Nonalcoholic Fatty Liver Disease Fibrosis Score in Identifying Advanced Fibrosis in Subjects With Nonalcoholic Fatty Liver Disease: A Meta-Analysis. *Am J Gastroenterol* 2021; **116**: 1833-1841 [PMID: 34160377 DOI: 10.14309/ajg.0000000000001337]

23   **McPherson S**, Hardy T, Dufour JF, Petta S, Romero-Gomez M, Allison M, Oliveira CP, Francque S, Van Gaal L, Schattenberg JM, Tiniakos D, Burt A, Bugianesi E, Ratziu V, Day CP, Anstee QM. Age as a Confounding Factor for the Accurate Non-Invasive Diagnosis of Advanced NAFLD Fibrosis. *Am J Gastroenterol* 2017; **112**: 740-751 [PMID: 27725647 DOI: 10.1038/ajg.2016.453]

ORIGINAL ARTICLE

Retrospective Study

Prognostic factors of recurrent intrahepatic cholangiocarcinoma after hepatectomy: A retrospective study

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Abstract

BACKGROUND

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour. Hepatectomy is an effective treatment for early ICC, but postoperative recurrence greatly affects patient survival. Studies on recurrent ICC after hepatectomy are lacking.

AIM

To investigate the clinical characteristics of patients with recurrent ICC after hepatectomy, analyse prognostic factors and explore diagnosis and treatment strategies.

METHODS

A retrospective analysis was performed on all ICC patients undergoing hepatectomy from January 2013 to August 2021. Patients with postoperative recurrence were selected according to the inclusion and exclusion criteria. Cumulative overall survival was plotted by the Kaplan-Meier method, and differences were assessed by univariate survival analysis using the log-rank test. Multivariate analysis of cumulative survival was performed using the Cox proportional risk model.

RESULTS

During the 8-year study period, 103 patients underwent ICC-related hepatectomy, and 54 exhibited postoperative recurrence. The median disease-free survival (DFS) was 6 mo, the median overall survival (OS) was 9 mo, and the cumulative OS rates at 1, 2 and 3 years after the operation were 40.7%, 14.8% and 7.4%,

respectively. The median OS after recurrence was 4 mo, and the cumulative OS rates at 1, 2 and 3 years after recurrence were 16.1%, 6.7% and 3.4%, respectively. Multivariate analysis showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, $P = 0.007$] and DFS < 6 mo (HR = 3.47, 95%CI: 1.59-7.60, $P = 0.002$) were independent risk factors for the cumulative survival of patients with recurrence, while treatment after recurrence (HR = 0.21, 95%CI: 0.08-0.55, $P = 0.001$) was an independent protective factor. The median OS time of patients receiving multimodality therapy after recurrence of ICC was 7 mo, which was significantly higher than that of patients receiving only local therapy (3 mo), patients receiving systematic therapy (4 mo) and patients receiving the best supportive therapy (1 mo). Patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not ($P = 0.026$).

CONCLUSION

The prognosis of patients with recurrence after ICC-related hepatectomy is poor. Alcohol consumption and DFS < 6 mo are independent risk factors in terms of the cumulative survival of patients with recurrence, while treatment after recurrence is an independent protective factor. Multimodality therapy can effectively improve the prognosis of patients.

Key Words: Intrahepatic cholangiocarcinoma; Hepatectomy; Recurrence; Multimodality therapy; Prognosis

Core Tip: With this 8-year retrospective study, we aimed to investigate the clinical characteristics, analyse the prognostic factors, and discuss therapeutic strategies for patients with recurrent intrahepatic cholangiocarcinoma (ICC) after hepatectomy. Multivariate analysis showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, $P = 0.007$] and disease-free survival < 6 mo (HR=3.47, 95%CI: 1.59-7.60, $P = 0.002$) were independent risk factors for cumulative survival for patients with recurrence, while treatment after recurrence (HR=0.21, 95%CI: 0.08-0.55, $P = 0.001$) was an independent protective factor. We propose that multimodality therapy should be developed to improve long-term outcomes through the combined approach of local therapy, chemotherapy, targeted therapy, and immunotherapy.

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour originating from intrahepatic bile duct epithelial cells[1]. Liver cancer ranks sixth in the world in terms of incidence rate and third in terms of mortality rate[2]. ICC accounts for 10% to 15% of primary liver cancers[1]. In the last 30 years, the incidence and mortality rates of ICC have significantly increased worldwide[3]. Hepatectomy is an effective method for the treatment of early ICC[4]. However, ICC has highly malignant biological behaviour, and early recurrence and metastasis are extremely common, so the prognosis is poor[5]. The postoperative 5-year survival rate is only 20%-35%, and the recurrence rate is as high as 50%-70%, and these rates are much worse than those for hepatocellular carcinoma[6,7].

Prevention of ICC recurrence and treatment strategies after recurrence are extremely important to improve the overall survival (OS) time. The early recurrence of ICC is related to the characteristics of the tumour, while late recurrence is related to underlying liver diseases[8]. Studies[9] have shown that the presence of multiple tumours, microvascular invasion, and lymph node metastasis are risk factors for recurrence after hepatectomy. Age, liver disease, lymph node involvement, vascular invasion, multiple tumours, and tumour size are related to prognosis[10]. However, the risk factors affecting the long-term prognosis of patients with recurrent ICC after hepatectomy are not clear. The European Association for Liver Research[11] and the Italian Clinical Practice Guide[12] have pointed out that the treatment strategy for recurrent ICC is based on the clinical characteristics of the site of tumour recurrence. Recently, some studies[6,13] have reported various treatments for different types of recurrence. However, the best treatment strategy for the postoperative recurrence of ICC is still unclear.

In this study, the clinical characteristics and treatment statistics of patients with recurrent ICC after hepatectomy in our hospital were assessed to identify survival-related factors and explore strategies for diagnosis and treatment.

MATERIALS AND METHODS

Patients

The clinical data of 103 ICC patients who underwent hepatectomy in the First Affiliated Hospital of Zhengzhou University were analyzed retrospectively from January 2013 to August 2021. The diagnosis of ICC was based on liver pathological examination, and histological grading was based on the WHO grading system[14]. The tumour stage was determined according to the American Joint Council on Cancer (AJCC) 8th edition tumour-node-metastasis classification system[15]. The inclusion criteria were as follows: (1) Primary intrahepatic cholangiocarcinoma was confirmed by postoperative histopathology; (2) Liver function was considered Child-Pugh grade A or B; (3) Preoperative evaluation indicated that the patient could tolerate surgery without serious heart, lung, brain, and kidney vital organ lesions; and (4) Relapse was observed after hepatectomy. The exclusion criteria were as follows: (1) The patient had a preoperative history of malignant tumour; (2) Postoperative histopathology confirmed hepatocellular carcinoma or mixed liver cancer; or (3) Clinical records and follow-up information were incomplete. Finally, a total of 54 patients with recurrent ICC after hepatectomy were included (Figure 1). The study was approved by the Scientific Research and Clinical Trial Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Ethical number 2021-KY-0464-001).

Treatment strategy for primary ICC-related hepatectomy

The mode of operation was determined according to the location and size of the tumour and the patient's liver function. The scope of resection was classified according to the international consensus standard[16]: Extended hepatectomy was performed in 16 cases (resection of liver tissue more than 3 segments), and local hepatectomy was performed in 38 cases (marginal partial hepatectomy or resection of liver tissue no more than 3 segments). Abnormal enlargement of lymph nodes was found during the operation or imaging examination before the operation, and the hepatic hilum, hepatoduodenal ligament, and posterior pancreatic lymph nodes were dissected. Lymph node dissection was performed in 19 of the 54 patients. According to National Comprehensive Cancer Network (NCCN) practice guidelines[17], 20 ICC patients were treated with adjuvant therapy after hepatectomy.

Follow-up and recurrence

After hepatectomy, all patients were followed up *via* outpatient visits or telephone calls. Follow-up was initiated 1 mo after intrahepatic cholangiocarcinoma resection, followed by follow-up visits every 3 mo for 2 years and every 6 mo after 2 years. Patients with postoperative recurrence of ICC were followed up once a month, and the last follow-up was in August 2021. During the follow-up period, the patient examinations included (1) Haematology examination, including assessment of liver and kidney function, serum tumour markers, and hepatitis viral load; and (2) Imaging examination, including chest plain film or nonenhanced CT, abdominal enhanced CT or MRI. To evaluate the progression of the disease, patients with recurrent ICC were examined by whole-body bone scan or PET-CT. Follow-up began at the time of hepatectomy and ended at the time of death or the last follow-up. Disease-free survival (DFS) was defined as the time from the date of surgery to the first recurrence of ICC. OS was defined as the time from the first recurrence after hepatectomy to death or the last follow-up.

Treatment strategies after relapse

For those who are diagnosed with tumour recurrence or metastasis, the treatment plan is determined according to the evaluation of the reserve function of the liver, the condition of the whole body, and the site of recurrence. The inclusion criteria of secondary hepatectomy were the same as those of primary hepatectomy. Patients with unresectable ICC are treated with local therapy, chemotherapy, targeted therapy, immunotherapy, and multimodality therapy.

Statistical methods

For descriptive statistics, continuous variables are expressed as medians, and categorical variables are expressed as numbers (%). Cumulative survival was plotted by the Kaplan-Meier method. The log-rank test was used to assess differences in the univariate survival analysis. Multivariate analysis of cumulative survival was performed using the Cox proportional risk model. Statistical analysis was performed using SPSS 26.0 software (IBM, Armonk, NY, United States). Differences were considered statistically significant at $P < 0.05$. An online tool (http://www.bioinformatics.com.cn) was applied to draw Venn digrams related to recurrent patterns.
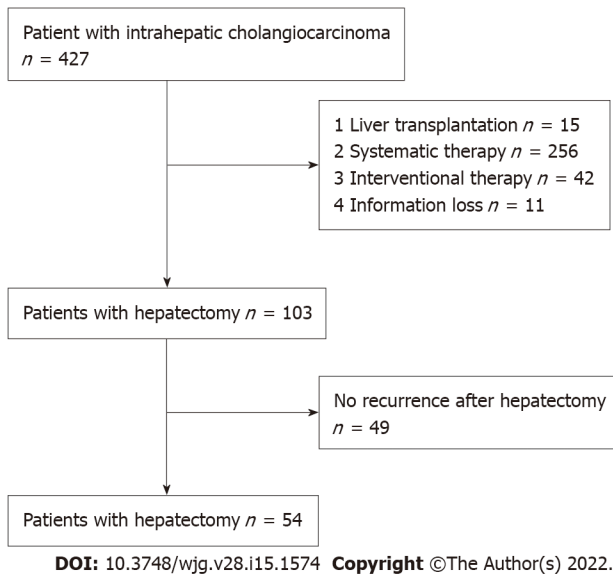
Figure 1 Patient flowchart.

RESULTS

Follow-up results and clinical characteristics of patients with recurrent ICC

By the end of follow-up, 54 ICC patients (54/103, 52.4%) had recurrence after hepatectomy. Patients were followed up for 2-94 mo, with a median DFS of 6 mo and a median OS of 9 mo. The 1-year, 2-year, and 3-year cumulative OS rates were 40.7%, 14.8%, and 7.4%, respectively (Figure 2). The median OS after recurrence was 4 mo, and the 1-year, 2-year and 3-year cumulative survival rates after recurrence were 16.1%, 6.7%, and 3.4%, respectively (Figure 3). The majority of patients (45/54, 83.3%) relapsed within 1 year, and the recurrence rate was 50% (27/54) within 6 mo after surgery. Venn diagrams showed that intrahepatic lesions (25/54, 46.3%) were the most common recurrence sites, followed by concurrent liver and lymph lesions (13/54, 24.1%) (Figure 4). The clinical and pathological features of patients with recurrence are shown in Table 1. Most of the patients were male (34/54, 63.0%), smokers (34/54, 63.0%), and alcohol consumers (44/54, 81.5%). Sixteen patients had hypertension, and 9 patients had diabetes. Twenty-four patients were associated with hepatitis B virus (HBV), and 9 patients associated with hepatitis C virus (HCV). Sixteen patients were treated with extensive hepatectomy, and lymph node dissection was performed in 19 patients. Postoperative pathological reports showed that 46 patients had single tumours, 22 patients had poorly differentiated tumours, and 12 patients had vascular tumour thrombi.

Prognostic factors in patients with ICC recurrence

According to the univariate analysis of patients with recurrent ICC, nine factors significantly affected the survival of patients (Table 1). Age, alcohol consumption, histological grade, biliary invasion, vascular tumour thrombi, DFS, preoperative and post-recurrence CA19-9 level, and treatment after recurrence were significant favorable prognostic indicators in patients with recurrent ICC. Multivariate Cox regression analysis showed that alcohol consumption, DFS < 6 mo and treatment after recurrence were independent factors affecting cumulative survival in patients with recurrence (Table 2). Early recurrence in ICC patients was associated with biliary invasion, vascular tumour thrombi, and high post-recurrence CA19-9 levels. Multivariate analysis proved that the risk of death from alcohol consumption was 4.64 times that of non-alcohol consumption, and this was independent of other prognostic factors. The mortality risk of patients with DFS < 6 mo was 3.47 times that of patients with DFS > 6 mo. Treatment after recurrence could significantly reduce the mortality risk.

Treatment after recurrence

Treatment patterns for patients with recurrent ICC are shown in Table 3. Fourteen patients received local therapy, 22 patients received systematic therapy, 6 patients received multimodality therapy, and 12 patients received supportive care therapy based on their condition. Figure 5 shows patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not (P = 0.026, log-rank test). Among the patients who received local treatment, 2 patients had hepatectomy after recurrence; 1 patient had received local liver resection, and hepatectomy was performed again after 7 mo. the patient died due to multiple metastases. Another patient underwent laparoscopic hepatectomy for the primary lesion, but the tumour recurred 13 mo

Table 1 Univariate analysis of prognostic factors after intrahepatic cholangiocarcinoma recurrence following hepatectomy

Factors	Cases, *n* (%)	Median survival after recurrence, mo (95%CI)	*P* value[1]
Sex			0.123
Male	34 (63.0)	4.0 (1.2-6.8)	
Female	20 (37.0)	3.0 (1.1-4.9)	
Age (yr)			0.031
< 65	42 (77.8)	4.0 (2.3-5.8)	
≥ 65	12 (22.2)	3.0 (1.3-5.1)	
Smoking			0.059
No	20 (37.0)	5.0 (2.0-8.0)	
Yes	34 (63.0)	3.0 (1.0-4.1)	
Alcohol consumption			0.004
No	10 (18.5)	10.0 (3.0-17.0)	
Yes	44 (81.5)	3.0 (1.9-4.2)	
Hypertension			0.309
No	38 (70.4)	4.0 (2.4-5.6)	
Yes	16 (29.6)	4.0 (0.0-11.3)	
Diabetes			0.193
No	45 (83.3)	4.0 (2.5-5.5)	
Yes	9 (16.7)	8.0 (1.0-15.0)	
Hepatitis B			0.904
Negative	30 (55.6)	3.0 (1.3-4.7)	
Positive	24 (44.4)	5.0 (2.0-8.0)	
Hepatitis C			0.175
Negative	45 (83.3)	4 .0 (2.2-5.8)	
Positive	9 (16.7)	5.0 (2.1-7.9)	
Anti-hepatitis-virus			0.969
No	29 (53.7)	4.0 (2.4-5.6)	
Yes	25 (46.3)	5.0 (1.9-8.1)	
Cholelithiasis			0.181
No	44 (81.5)	4.0 (2.1-5.9)	
Yes	10 (18.5)	2.0 (0.0-4.8)	
CA19-9 (U/mL) (initial)			0.002
< 200	33 (61.1)	5.0 (1.8-8.2)	
≥ 200	21 (38.9)	2.0 (1.3-2.7)	
CEA (ng/mL) (initial)			0.356
< 5	34 (63.0)	4.0 (2.1-5.9)	
≥ 5	20 (37.0)	3.0 (0.8-5.2)	
Hepatectomy			0.855
Limited	38 (70.4)	4.0 (2.1-5.9)	
Extended	16 (29.6)	3 .0 (0.0-6.9)	
Lymph node dissection			0.909

No	35 (64.8)	4.0 (2.2-5.8)	
Yes	19 (35.2)	5.0 (2.1-7.9)	
Adjuvant treatment			0.619
No	34 (63.0)	3 .0 (0.1-5.9)	
Yes	20 (37.0)	4 .0 (2.7-5.3)	
Tumor size (cm)			0.884
< 5	14 (25.9)	4.0 (0.4-7.6)	
≥ 5	40 (74.1)	4 .0 (2.0-6.0)	
Multiplicity			0.803
Solitary	46 (85.2)	4.0 (1.4-6.6)	
Multiple	8 (14.8)	3.0 (1.3-4.7)	
Satellite nodules			0.953
No	34 (63.0)	4.0 (2.1-5.9)	
Yes	20 (37.0)	4.0 (2.4-5.6)	
Histological grade			0.021
PD	22 (40.7)	2.0 (1.2-2.8)	
WD or MD	32 (59.3)	5.0 (3.5-6.5)	
Vascular invasion			0.705
No	49 (90.7)	4.0 (2.1-5.9)	
Yes	5 (9.3)	5.0 (1.7-8.3)	
Lymph node metastasis			0.531
No	29 (53.7)	3.0 (0.9-5.1)	
Yes	25 (46.3)	5.0 (2.6-7.4)	
Perineural invasion			0.428
No	45 (83.3)	4.0 (2.5-5.5)	
Yes	9 (16.7)	2.0 (1.3-2.7)	
Biliary invasion			0.003
No	35 (64.8)	5.0 (2.6-7.4)	
Yes	19 (35.2)	2.0 (1.2-2.8)	
Vascular tumour thrombi			0.002
No	42 (77.8)	5.0 (3.2-6.8)	
Yes	12 (22.2)	2.0 (1.2-2.6)	
AJCC T category			0.196
T1–2	14 (25.9)	4.0 (0.0-11.3)	
T3–4	40 (74.1)	4.0 (2.1-5.9)	
DFS			0.003
< 6 mo	27 (50.0)	2.0 (0.7-3.3)	
≥ 6 mo	27 (50.0)	6.0 (2.3-9.7)	
CA19-9 (U/mL) (recurrence)			0.002
< 200	35 (64.8)	4.0 (1.3-6.7)	
≥ 200	19 (35.2)	2.0 (1.2-2.8)	
CEA (ng/mL) (recurrence)			0.378
< 5	41 (75.9)	4.0 (2.3-5.7)	

≥ 5	13 (24.1)	7.0 (1.7-12.3)	
NLR (recurrence)			0.804
< 2	24 (44.4)	5.0 (2.8-7.2)	
≥ 2	30 (55.6)	2.0 (0.9-3.1)	
Recurrent site			0.334
Intrahepatic	24 (44.4)	4.0 (2.3-5.7)	
Extrahepatic	6 (11.2)	3.0 (0.0-11.6)	
Intrahepatic + extrahepatic	24 (44.4)	2.0 (0.4-3.6)	
Treatment after recurrence			< 0.001
No	12 (22.2)	2.0 (1.0-2.8)	
Yes	42 (77.8)	5.0 (2.9-7.1)	

[1]Data are based on log-rank test.

N: Number; CI: Confidence interval; CA19-9: Serum carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; PD: Poor-differentiated; WD: Well-differentiated; MD: Moderate differentiated; NLR: Neutrophil-to-lymphocyte ratio; AJCC: the American Joint Committee on Cancer; DFS: Disease free survival.

Table 2 Multivariate analysis of prognostic factors after intrahepatic cholangiocarcinoma recurrence following hepatectomy

Factors	Multivariate analysis		
	HR	95%CI	*P* value[1]
Age (≥ 65 yr)	2.12	0.88-5.12	0.096
Alcohol consumption (Yes)	4.64	1.53-14.04	0.007
CA19-9 (≥ 200 U/mL) (initial)	2.63	0.94-7.35	0.065
Histological grade (PD)	1.18	0.59-2.35	0.646
Biliary invasion (Yes)	1.04	0.41-2.63	0.940
Vascular tumor thrombus (Yes)	1.80	0.70-4.65	0.222
DFS (< 6 mo)	3.47	1.59-7.60	0.002
CA19-9 (≥ 200 U/mL) (recurrence)	1.13	0.47-2.71	0.785
Treatment after recurrence (Yes)	0.21	0.08-0.55	0.001

[1]Data are based on Cox regression model.

CI: Confidence interval; CA19-9: Serum carbohydrate antigen 19-9; PD: Poor-differentiated; DFS: Disease free survival.

after surgery. Local hepatectomy and lymph node dissection were performed for the recurrent lesion. Table 4 shows the clinicopathological features of the 6 patients who received multimodality therapy for recurrence, of which 2 patients (No. 4, 6) survived, and 4 patients (No. 1, 2, 3, 5) died due to tumour-related complications. Three patients with recurrence (No. 1, 2, 3) received the GEMOX regimen (1 g/m² gemcitabine on d 1 and 8 + 100 mg/m² oxaliplatin on d 1 with 21 d/cycle) after transarterial chemoembolization (TACE). One patient (No. 4) received local therapy after intrahepatic recurrence. Due to extrahepatic metastasis, the patient was switched to the SOX regimen (60 mg/d tegafur on d 1-14 + 130 mg/m² oxaliplatin on d 1 with 21 d/cycle) maintenance therapy. One patient with recurrence (No. 5) received the FOLFOX-4 regimen (400 mg/m² fluorouracil on d 1 and 2 + 200 mg/m² calcium folate on d 1 and 2 + 85 mg/m² oxaliplatin on d 1 with 14 d/cycle) after RFA. One patient (No. 6) received a tyrosine kinase inhibitor regimen + PD-1 inhibitor (250 mg/d apatinib mesylate + 200 mg camrelizumab on d 1 with 21 d/cycle) after RFA, and no disease progression was observed up to the submission date.

DISCUSSION

ICC is a rare invasive biliary tract tumour and primary liver malignancy with an increasing incidence worldwide[3]. Most patients are initially diagnosed with advanced ICC, and only 30% of ICCs can be

Table 3 Treatment of recurrent intrahepatic cholangiocarcinoma

Treatment	n	Median DFS, mo (range)	MS after recurrence, mo (range)
Local therapy	14	6 (1-86)	3 (1-36)
Hepatectomy	2	10 (7-13)	25 (13-36)
TACE	8	3 (1-23)	2 (1-7)
RFA	4	7 (2-86)	3 (1-7)
Systemic therapy	22	5 (1-29)	4 (1-38)
Chemotherapy	3	11 (10-29)	4 (1-38)
Targeted therapy	12	3 (1-23)	4 (1-10)
Targeted + immunization therapy	7	4 (1-13)	7 (2-24)
Multimodality therapy	6	4 (2-11)	7 (4-24)
Best supportive care	12	6 (3-20)	1 (1-6)

N: Number; DFS: Disease-free survival; MS: Median survival; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation.

Table 4 Individual characteristics of patients receiving multimodality therapy for recurrent intrahepatic cholangiocarcinoma

Clinical features					Pathological characteristics				Recurrent		Survival		
Case	Age (yr)	Sex	Hepatitis	Surgery	Lymph node metastasis	Tumour size (cm)	Tumour number	Histological grade	Recurrent site	Treatment	DFS (mo)	SAR (mo)	Outcome
1	62	M	HBV	ERH	Yes	12	1	MD	Liver + lymph node	TACE + GEMOX	4	3	Dead
2	48	M	HBV	ERH	None	8	1	MD	Liver	TACE + GEMOX	11	13	Dead
3	40	M	HBV	ERH	None	5	2	MD	Liver + lymph node + bone	TACE + GEMOX	2	5	Dead
4	53	M	None	LH	None	11	1	MD	Liver + lymph node	RFA + TACE + SOX	6	6	Alive
5	44	M	HBV	RH	None	14	1	MD	Liver + bone	RFA + FOLFOX-4	3	2	Dead
6	64	M	None	ERH	Yes	22	1	MD	Liver	RFA + PD-1 + TKI	4	1	Alive

DFS: Disease-free survival; SAR: Survival after recurrence; M: Male; HBV: Hepatitis B virus; ERH: Extended right hepatectomy; LH: Left hepatectomy; RH: Right hepatectomy; MD: Moderate differentiated; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; GEMOX: Gemcitabine + oxaliplatin; FOLFOX-4: Fluorouracil + calcium folate + oxaliplatin; SOX: Tegafur + oxaliplatin; PD-1: Programmed death-1 inhibitor; TKI: Tyrosine kinase inhibitor.

surgically resected[18]. Surgical principles include negative margins and tumour-related lymph node resection[19-21]. Due to the biological characteristics of ICC and the vascular system of the liver, local recurrence and lymphatic metastasis are highly likely to occur after surgery. Even after R0 resection, the recurrence rate 5 years after hepatectomy is as high as 70%[6]. At present, the choice of treatment for recurrent ICC remains controversial. Since ICC after recurrence seriously affects the postoperative survival of patients, it is necessary to determine the risk factors for survival after recurrence and explore diagnosis and treatment strategies.

Previous studies[22,23] have explored the clinical characteristics and prognostic factors of the postoperative recurrence of ICC, but few studies have shown the prognosis of ICC patients after recurrence. Chan *et al*[22] reported that tumour diameter > 5 cm, tumour type, lymph node invasion, and vascular invasion are independent risk factors for recurrence in patients after hepatectomy. In other studies, Addeo *et al*[23] found that the risk factors influencing patient recurrence were related to the degree of tumour differentiation and the number of tumours. Regarding the prognosis of patients with recurrent ICC, Ohira *et al*[10] reported that tumour type and nonsurgical treatment were related to a
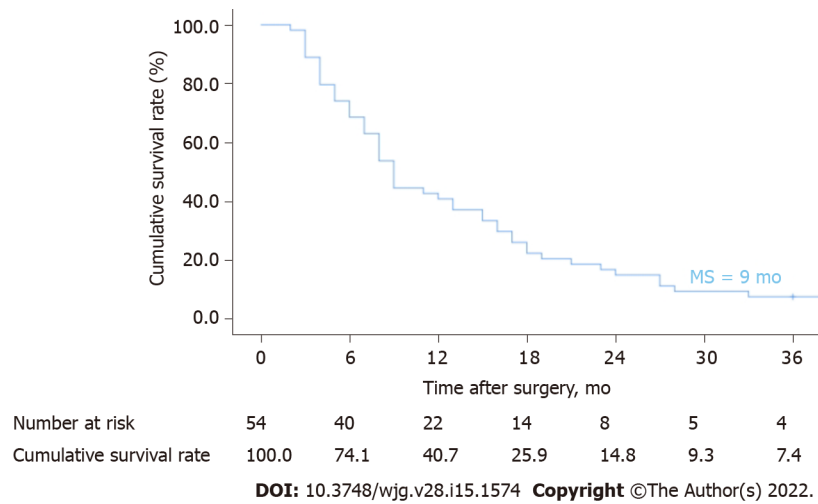
Figure 2 Kaplan-Meier curves of ICC recurrence: Time after surgery. ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.
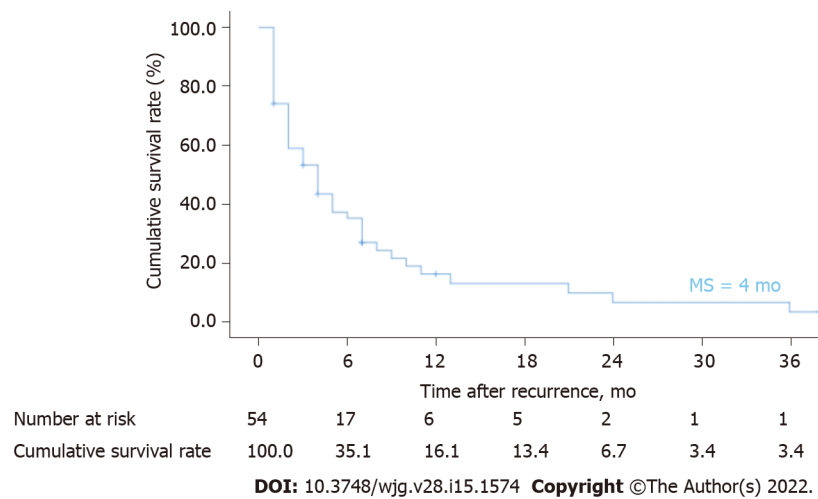
Figure 3 Kaplan-Meier curves of ICC recurrence: Time after recurrence. ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.

poor prognosis. Our study found that alcohol consumption and DFS < 6 mo were independent risk factors affecting the cumulative survival rate of patients with recurrence, and treatment after recurrence was an independent protective factor.

Previous studies[24] have shown that alcohol consumption is a risk factor for ICC. Alcohol may interfere with DNA synthesis and repair through the mechanism of acetaldehyde, a product of ethanol oxidation, to promote the occurrence of liver cancer[25]. Although alcohol drinking is associated with the aetiology of ICC, it is not clear whether alcohol drinking affects the prognosis and survival of patients with recurrent ICC. In this study, multivariate analysis showed that alcohol consumption may be an independent risk factor for recurrent ICC. For patients with recurrent ICC, we recommend reducing alcohol consumption as much as possible to improve the prognosis and survival time of patients.

In this study, the first recurrence of most patients after hepatectomy occurred within 1 year after surgery. Multivariate analysis showed that DFS < 6 was an independent risk factor for survival after ICC recurrence. Park *et al*[26] and Si *et al*[27] also reported that DFS was associated with prognosis. Compared with the clinical characteristics of patients with advanced recurrence, early recurrence is often accompanied by bile duct invasion and lymph node metastasis, and the median survival time after recurrence is 2 mo, which is much lower than the time of late recurrence. Currently, immunohisto-chemical markers commonly used to predict early recurrence after hepatectomy include B-lymphocyte chemokine 13 (CXCL13)[28], pancreatic secreted trypsin inhibitor (PSTI)[29], and insulin-like growth factor-II mRNA binding protein 3 (IMP3)[30]. Even if the prognosis of patients with early recurrence of ICC after primary hepatectomy is poor, surgical treatment should be considered to improve the prognosis.
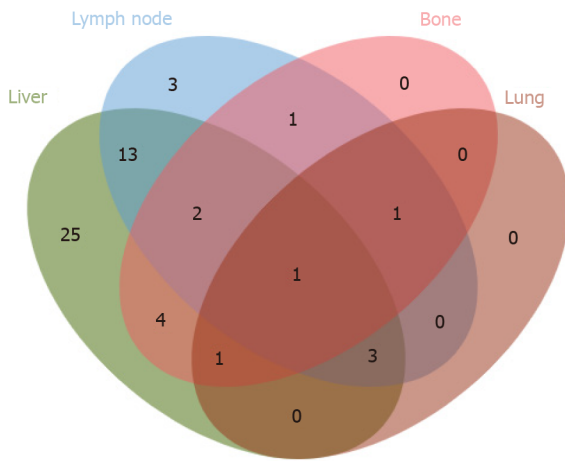
Figure 4 Venn diagram of recurrent location pattern. Intrahepatic lesions (25/54, 46.3%) were the most common recurrence sites, followed by concurrent liver and lymph nodes (13/54, 24.1%).
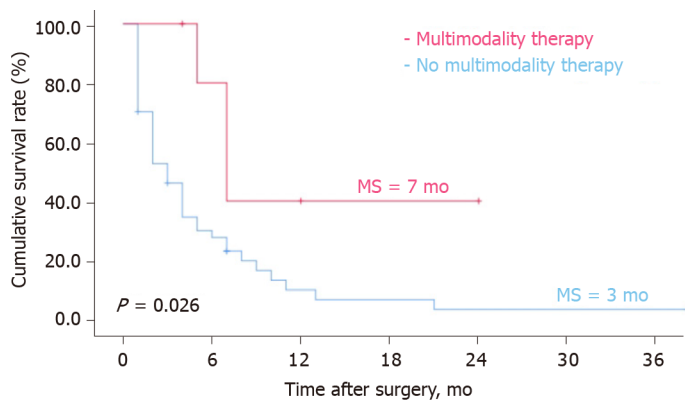
| Number at risk | 6 | 4 | 1 | 1 | 0 | 0 | 0 |
| Cumulative survival rate | 48 | 12 | 1 | 1 | 1 | 1 | 1 |

Figure 5 Kaplan-Meier curves of ICC recurrence: multimodality therapy versus no multimodality therapy. Patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not (P = 0.026, log-rank test). ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.

The survival time of patients with recurrent ICC after surgical resection is higher than that of patients without surgical resection. Furthermore, compared with other treatments, secondary hepatectomy significantly improved the OS time of patients with recurrent ICC. Studies[31] have shown that the prognosis of recurrent intrahepatic resection of ICC is comparable to that of primary resection. In a multicentre study of 356 patients with ICC who underwent hepatectomy, approximately 60% exhibited postoperative recurrence, and 37 of them underwent reresection, with a 5-year survival rate of 44%[32]. Recent studies[33] have reported that repeat resection after recurrence significantly prolongs OS compared with palliative treatment. Therefore, we suggest that patients with resectable intrahepatic recurrent ICC can undergo reoperation to improve patient outcomes.

Most recurrent ICCs are highly invasive and have limitations, such as insufficient remaining liver, making patients ineligible for secondary hepatectomy. Multimodality therapies include strategies that combine regional therapy, systemic chemotherapy, targeted therapy, and immunotherapy. In this study, 6 patients with recurrent ICC who were mainly treated with multimodality therapies achieved a higher postoperative median OS (7 mo) than those with local treatment (3 mo), systemic treatment (4 mo), and supportive treatment (1 mo).

Systemic chemotherapy combined with local therapy can significantly improve patient prognosis. Intra-arterial therapies combined with chemotherapy can shrink the lesion to achieve R0 resection[34]. In this study, the median survival of 3 patients with recurrent ICC treated with TACE combined with the GEMOX regimen was 5 mo, which was longer than that of patients with recurrent ICC using chemotherapy alone (median OS, 4 mo). RFA is suitable for local tumours with diameters < 5 cm, and tumour numbers < 3. RFA was superior to systematic chemotherapy in this study[35]. Among the 6

patients with recurrent ICC, 2 patients underwent RFA combined with chemotherapy. One patient had recurrence 3 mo after hepatectomy, and local intrahepatic lesions were treated with RFA combined with the FOLFOX-4 regimen. The other patient relapsed 6 mo after surgery, and 2 mo after RFA, multiple intrahepatic metastases occurred. TACE combined with the SOX regimen was performed again. Currently, the patient is still alive. A new approach of radiotherapy combined with chemotherapy in the open treatment of advanced ICC. Studies[36] have shown that radiotherapy with chemotherapy can not only relieve pain and other complications in patients with advanced ICC but can also improve the disease control rate and patient survival time. Japanese researchers[37] found that 60% of patients with advanced ICC underwent radical hepatocellular carcinoma after radiotherapy combined with systemic chemotherapy, and the 5-year survival rate was 24%. Radiotherapy was not included in our treatment strategy for patients with recurrent ICC. Due to the lack of reliable evidence-based medical data, the NCCN practice guidelines[17] did not recommend radiotherapy as routine treatment for recurrent ICC.

Among the 6 patients with recurrent ICC in our centre, one patient was treated with a tumour immune checkpoint inhibitor combined with targeted therapy after RFA; this patient was still alive without disease progression at the time of submission. PD-L1 expression was found in interstitial cells in 30% of ICC patients[38]. In the tumour microenvironment of connective tissue hyperplasia and immune system deficiency in ICC, the clinical efficacy of a single drug PD-1/PD-L1 inhibitor in tumour suppression is poor[39]. Targeted therapy combined with immunotherapy is being explored[40]. Targeted drugs can induce the death of tumour cells, leading to the release of their own antigens, which are then taken up by antigen-presenting cells to activate specific T cells. However, they also upregulate inhibitory factors such as CTLA-4 and PD-1. Therefore, the combination of PD-1 inhibitors can strengthen the killing effect, reduce the attack of nontumour antigens, and reduce the adverse reactions of immunotherapy[41]. The combination of tumour immune checkpoint inhibitors and targeted therapy is still a hotspot in the field of tumour therapy.

There are several limitations to this study. First, this is a retrospective study, and there may be selection and detection bias in patients with recurrent ICC. Second, ICC is a rare disease. Although the clinical study lasted for 8 years, the number of patients with recurrence is small, and there are not enough randomized controlled trials of recurrent patients. Finally, this is a single-centre study, so multicentre and prospective trials are needed to confirm our results.

CONCLUSION

The prognosis of patients with recurrence after ICC-related hepatectomy is poor. Alcohol consumption and DFS < 6 mo are independent risk factors in terms of the cumulative survival of patients with recurrence, while treatment after recurrence is an independent protective factor. We propose that multimodality therapy should be developed to improve long-term outcomes through the combined approach of local therapy, chemotherapy, targeted therapy, and immunotherapy.

ARTICLE HIGHLIGHTS

Research background
Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour originating from intrahepatic bile duct epithelial cells. Recurrence is very common after hepatectomy.

Research motivation
There are few reports on the clinical features and prognostic factors of recurrent ICC, and the treatment strategies for recurrent ICC have not been fully clarified.

Research objectives
The objective of this study was to analyze the prognostic factors of recurrent ICC and to explore treatment strategies.

Research methods
We retrospectively analyzed all ICC patients who underwent hepatectomy at the First Affiliated Hospital of Zhengzhou University between January 2013 and August 2021. We summarized the clinical characteristics of patients with recurrent ICC and assessed prognostic factors by univariate and multivariate analyses.

Research results
Recurrence occurred in 54 of 103 patients with ICC after hepatectomy during the study period. The median OS of patients with recurrent ICC was 4 mo, and the cumulative OS rates at 1, 2, and 3 years

after recurrence were 16.1%, 6.7%, and 3.4%, respectively. Multivariate analysis of cumulative survival by the Cox proportional risk model showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, P = 0.007], DFS < 6 mo (HR = 3.47, 95%CI: 1.59-7.60, P = 0.002) and treatment after recurrence (HR = 0.21, 95%CI: 0.08-0.55, P = 0.001) were independent factors for recurrence. Patients who received multimodality therapy had higher survival rates than those who did not (P = 0.026).

Research conclusions

The prognosis of recurrent patients is related to alcohol consumption, DFS < 6 mo and treatment after recurrence. Active and effective multidisciplinary treatment is beneficial to improve the prognosis of patients.

Research perspectives

Multicentre prospective studies are needed to evaluate the efficacy of multidisciplinary treatment in recurrent ICC.

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REFERENCES

1   **Kelley RK**, Bridgewater J, Gores GJ, Zhu AX. Systemic therapies for intrahepatic cholangiocarcinoma. *J Hepatol* 2020; **72**: 353-363 [PMID: 31954497 DOI: 10.1016/j.jhep.2019.10.009]

2   **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; **71**: 209-249 [PMID: 33538338 DOI: 10.3322/caac.21660]

3   **Sirica AE**, Gores GJ, Groopman JD, Selaru FM, Strazzabosco M, Wei Wang X, Zhu AX. Intrahepatic

Cholangiocarcinoma: Continuing Challenges and Translational Advances. *Hepatology* 2019; **69**: 1803-1815 [PMID: 30251463 DOI: 10.1002/hep.30289]

4   **Zhang H**, Yang T, Wu M, Shen F. Intrahepatic cholangiocarcinoma: Epidemiology, risk factors, diagnosis and surgical management. *Cancer Lett* 2016; **379**: 198-205 [PMID: 26409434 DOI: 10.1016/j.canlet.2015.09.008]

5   **Zhou R**, Lu D, Li W, Tan W, Zhu S, Chen X, Min J, Shang C, Chen Y. Is lymph node dissection necessary for resectable intrahepatic cholangiocarcinoma? *HPB (Oxford)* 2019; **21**: 784-792 [PMID: 30878490 DOI: 10.1016/j.hpb.2018.12.011]

6   **Hu LS**, Zhang XF, Weiss M, Popescu I, Marques HP, Aldrighetti L, Maithel SK, Pulitano C, Bauer TW, Shen F, Poultsides GA, Soubrane O, Martel G, Koerkamp BG, Itaru E, Pawlik TM. Recurrence Patterns and Timing Courses Following Curative-Intent Resection for Intrahepatic Cholangiocarcinoma. *Ann Surg Oncol* 2019; **26**: 2549-2557 [PMID: 31020501 DOI: 10.1245/s10434-019-07353-4]

7   **Doussot A**, Gonen M, Wiggers JK, Groot-Koerkamp B, DeMatteo RP, Fuks D, Allen PJ, Farges O, Kingham TP, Regimbeau JM, D'Angelica MI, Azoulay D, Jarnagin WR. Recurrence Patterns and Disease-Free Survival after Resection of Intrahepatic Cholangiocarcinoma: Preoperative and Postoperative Prognostic Models. *J Am Coll Surg* 2016; **223**: 493-505.e2 [PMID: 27296525 DOI: 10.1016/j.jamcollsurg.2016.05.019]

8   **Yoh T**, Hatano E, Seo S, Okuda Y, Fuji H, Ikeno Y, Taura K, Yasuchika K, Okajima H, Kaido T, Uemoto S. Long-Term Survival of Recurrent Intrahepatic Cholangiocarcinoma: The Impact and Selection of Repeat Surgery. *World J Surg* 2018; **42**: 1848-1856 [PMID: 29218465 DOI: 10.1007/s00268-017-4387-7]

9   **Cholangiocarcinoma Working Group**. Italian Clinical Practice Guidelines on Cholangiocarcinoma - Part I: Classification, diagnosis and staging. *Dig Liver Dis* 2020; **52**: 1282-1293 [PMID: 32893173 DOI: 10.1016/j.dld.2020.06.045]

10   **Ohira M**, Kobayashi T, Hashimoto M, Tazawa H, Abe T, Oshita A, Kohashi T, Irei T, Oishi K, Ohdan H; Hiroshima Surgical study group of Clinical Oncology (HiSCO). Prognostic factors in patients with recurrent intrahepatic cholangiocarcinoma after curative resection: A retrospective cohort study. *Int J Surg* 2018; **54**: 156-162 [PMID: 29730072 DOI: 10.1016/j.ijsu.2018.04.058]

11   **Bridgewater J**, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014; **60**: 1268-1289 [PMID: 24681130 DOI: 10.1016/j.jhep.2014.01.021]

12   **Cholangiocarcinoma Working Group**. Italian Clinical Practice Guidelines on Cholangiocarcinoma - Part II: Treatment. *Dig Liver Dis* 2020; **52**: 1430-1442 [PMID: 32952071 DOI: 10.1016/j.dld.2020.08.030]

13   **Bartsch F**, Paschold M, Baumgart J, Hoppe-Lotichius M, Heinrich S, Lang H. Surgical Resection for Recurrent Intrahepatic Cholangiocarcinoma. *World J Surg* 2019; **43**: 1105-1116 [PMID: 30523392 DOI: 10.1007/s00268-018-04876-x]

14   **Nagtegaal ID**, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, Washington KM, Carneiro F, Cree IA; WHO Classification of Tumours Editorial Board. The 2019 WHO classification of tumours of the digestive system. *Histopathology* 2020; **76**: 182-188 [PMID: 31433515 DOI: 10.1111/his.13975]

15   **Amin MB**, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR, Winchester DP. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin* 2017; **67**: 93-99 [PMID: 28094848 DOI: 10.3322/caac.21388]

16   **Wakabayashi G**, Cherqui D, Geller DA, Buell JF, Kaneko H, Han HS, Asbun H, O'Rourke N, Tanabe M, Koffron AJ, Tsung A, Soubrane O, Machado MA, Gayet B, Troisi RI, Pessaux P, Van Dam RM, Scatton O, Abu Hilal M, Belli G, Kwon CH, Edwin B, Choi GH, Aldrighetti LA, Cai X, Cleary S, Chen KH, Schön MR, Sugioka A, Tang CN, Herman P, Pekolj J, Chen XP, Dagher I, Jarnagin W, Yamamoto M, Strong R, Jagannath P, Lo CM, Clavien PA, Kokudo N, Barkun J, Strasberg SM. Recommendations for laparoscopic liver resection: a report from the second international consensus conference held in Morioka. *Ann Surg* 2015; **261**: 619-629

17   **Benson AB**, D'Angelica MI, Abbott DE, Anaya DA, Anders R, Are C, Bachini M, Borad M, Brown D, Burgoyne A, Chahal P, Chang DT, Cloyd J, Covey AM, Glazer ES, Goyal L, Hawkins WG, Iyer R, Jacob R, Kelley RK, Kim R, Levine M, Palta M, Park JO, Raman S, Reddy S, Sahai V, Schefter T, Singh G, Stein S, Vauthey JN, Venook AP, Yopp A, McMillian NR, Hochstetler C, Darlow SD. Hepatobiliary Cancers, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* 2021; **19**: 541-565 [PMID: 34030131 DOI: 10.6004/jnccn.2021.0022]

18   **Weber SM**, Ribero D, O'Reilly EM, Kokudo N, Miyazaki M, Pawlik TM. Intrahepatic cholangiocarcinoma: expert consensus statement. *HPB (Oxford)* 2015; **17**: 669-680 [PMID: 26172134 DOI: 10.1111/hpb.12441]

19   **Li MX**, Bi XY, Li ZY, Huang Z, Han Y, Zhao JJ, Zhao H, Cai JQ. Impaction of surgical margin status on the survival outcome after surgical resection of intrahepatic cholangiocarcinoma: a systematic review and meta-analysis. *J Surg Res* 2016; **203**: 163-173 [PMID: 27338547 DOI: 10.1016/j.jss.2016.02.012]

20   **Zhang XF**, Chen Q, Kimbrough CW, Beal EW, Lv Y, Chakedis J, Dillhoff M, Schmidt C, Cloyd J, Pawlik TM. Lymphadenectomy for Intrahepatic Cholangiocarcinoma: Has Nodal Evaluation Been Increasingly Adopted by Surgeons over Time? *J Gastrointest Surg* 2018; **22**: 668-675 [PMID: 29264768 DOI: 10.1007/s11605-017-3652-2]

21   **Zhang XF**, Xue F, Dong DH, Weiss M, Popescu I, Marques HP, Aldrighetti L, Maithel SK, Pulitano C, Bauer TW, Shen F, Poultsides GA, Soubrane O, Martel G, Koerkamp BG, Itaru E, Lv Y, Pawlik TM. Number and Station of Lymph Node Metastasis After Curative-intent Resection of Intrahepatic Cholangiocarcinoma Impact Prognosis. *Ann Surg* 2021; **274**: e1187-e1195 [PMID: 31972643 DOI: 10.1097/SLA.0000000000003788]

22   **Chan KM**, Tsai CY, Yeh CN, Yeh TS, Lee WC, Jan YY, Chen MF. Characterization of intrahepatic cholangiocarcinoma after curative resection: outcome, prognostic factor, and recurrence. *BMC Gastroenterol* 2018; **18**: 180 [PMID: 30514231 DOI: 10.1186/s12876-018-0912-x]

23   **Addeo P**, Jedidi I, Locicero A, Faitot F, Oncioiu C, Onea A, Bachellier P. Prognostic Impact of Tumor Multinodularity in Intrahepatic Cholangiocarcinoma. *J Gastrointest Surg* 2019; **23**: 1801-1809 [PMID: 30478531 DOI: 10.1007/s11605-018-4052-y]

24   **Gupta A**, Dixon E. Epidemiology and risk factors: intrahepatic cholangiocarcinoma. *Hepatobiliary Surg Nutr* 2017; **6**: 101-104 [PMID: 28503557 DOI: 10.21037/hbsn.2017.01.02]

25   **Seitz HK**, Stickel F. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol Chem* 2006; **387**: 349-360 [PMID: 16606331 DOI: 10.1515/BC.2006.047]

26   **Park HM**, Yun SP, Lee EC, Lee SD, Han SS, Kim SH, Park SJ. Outcomes for Patients with Recurrent Intrahepatic Cholangiocarcinoma After Surgery. *Ann Surg Oncol* 2016; **23**: 4392-4400 [PMID: 27581609 DOI: 10.1245/s10434-016-5454-2]

27   **Si A**, Li J, Xing X, Lei Z, Xia Y, Yan Z, Wang K, Shi L, Shen F. Effectiveness of repeat hepatic resection for patients with recurrent intrahepatic cholangiocarcinoma: Factors associated with long-term outcomes. *Surgery* 2017; **161**: 897-908 [PMID: 27989605 DOI: 10.1016/j.surg.2016.10.024]

28   **Li C**, Kang D, Sun X, Liu Y, Wang J, Gao P. The Effect of C-X-C Motif Chemokine 13 on Hepatocellular Carcinoma Associates with Wnt Signaling. *Biomed Res Int* 2015; **2015**: 345413 [PMID: 26161394 DOI: 10.1155/2015/345413]

29   **Tonouchi A**, Ohtsuka M, Ito H, Kimura F, Shimizu H, Kato M, Nimura Y, Iwase K, Hiwasa T, Seki N, Takiguchi M, Miyazaki M. Relationship between pancreatic secretory trypsin inhibitor and early recurrence of intrahepatic cholangiocarcinoma following surgical resection. *Am J Gastroenterol* 2006; **101**: 1601-1610 [PMID: 16863567 DOI: 10.1111/j.1572-0241.2006.00612.x]

30   **Chen YL**, Jeng YM, Hsu HC, Lai HS, Lee PH, Lai PL, Yuan RH. Expression of insulin-like growth factor II mRNA-binding protein 3 predicts early recurrence and poor prognosis in intrahepatic cholangiocarcinoma. *Int J Surg* 2013; **11**: 85-91 [PMID: 23246869 DOI: 10.1016/j.ijsu.2012.11.021]

31   **Zhang XF**, Beal EW, Bagante F, Chakedis J, Weiss M, Popescu I, Marques HP, Aldrighetti L, Maithel SK, Pulitano C, Bauer TW, Shen F, Poultsides GA, Soubrane O, Martel G, Koerkamp BG, Itaru E, Pawlik TM. Early *vs* late recurrence of intrahepatic cholangiocarcinoma after resection with curative intent. *Br J Surg* 2018; **105**: 848-856 [PMID: 29193010 DOI: 10.1002/bjs.10676]

32   **Yamashita YI**, Shirabe K, Beppu T, Eguchi S, Nanashima A, Ohta M, Ueno S, Kondo K, Kitahara K, Shiraishi M, Takami Y, Noritomi T, Okamoto K, Ogura Y, Baba H, Fujioka H. Surgical management of recurrent intrahepatic cholangiocarcinoma: predictors, adjuvant chemotherapy, and surgical therapy for recurrence: A multi-institutional study by the Kyushu Study Group of Liver Surgery. *Ann Gastroenterol Surg* 2017; **1**: 136-142 [PMID: 29863136 DOI: 10.1002/ags3.12018]

33   **Kitano Y**, Yamashita YI, Nakagawa S, Okabe H, Imai K, Chikamoto A, Baba H. Effectiveness of surgery for recurrent cholangiocarcinoma: A single center experience and brief literature review. *Am J Surg* 2020; **219**: 175-180 [PMID: 30797594 DOI: 10.1016/j.amjsurg.2019.02.015]

34   **Wang L**, Lin ZG, Ke Q, Lou JY, Zheng SG, Bi XY, Wang JM, Guo W, Li FY, Wang J, Zheng YM, Li JD, Cheng S, Zhou WP, Zeng YY. Adjuvant transarterial chemoembolization following radical resection for intrahepatic cholangiocarcinoma: A multi-center retrospective study. *J Cancer* 2020; **11**: 4115-4122 [PMID: 32368294 DOI: 10.7150/jca.40358]

35   **Wu L**, Tsilimigras DI, Farooq A, Hyer JM, Merath K, Paredes AZ, Mehta R, Sahara K, Shen F, Pawlik TM. Potential survival benefit of radiofrequency ablation for small solitary intrahepatic cholangiocarcinoma in nonsurgically managed patients: A population-based analysis. *J Surg Oncol* 2019; **120**: 1358-1364 [PMID: 31614000 DOI: 10.1002/jso.25736]

36   **Hong TS**, Wo JY, Yeap BY, Ben-Josef E, McDonnell EI, Blaszkowsky LS, Kwak EL, Allen JN, Clark JW, Goyal L, Murphy JE, Javle MM, Wolfgang JA, Drapek LC, Arellano RS, Mamon HJ, Mullen JT, Yoon SS, Tanabe KK, Ferrone CR, Ryan DP, DeLaney TF, Crane CH, Zhu AX. Multi-Institutional Phase II Study of High-Dose Hypofractionated Proton Beam Therapy in Patients With Localized, Unresectable Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *J Clin Oncol* 2016; **34**: 460-468 [PMID: 26668346 DOI: 10.1200/JCO.2015.64.2710]

37   **Sumiyoshi T**, Shima Y, Okabayashi T, Negoro Y, Shimada Y, Iwata J, Matsumoto M, Hata Y, Noda Y, Sui K, Sueda T. Chemoradiotherapy for Initially Unresectable Locally Advanced Cholangiocarcinoma. *World J Surg* 2018; **42**: 2910-2918 [PMID: 29511872 DOI: 10.1007/s00268-018-4558-1]

38   **Kitano Y**, Yamashita YI, Nakao Y, Itoyama R, Yusa T, Umezaki N, Tsukamoto M, Yamao T, Miyata T, Nakagawa S, Okabe H, Imai K, Chikamoto A, Ishiko T, Baba H. Clinical Significance of PD-L1 Expression in Both Cancer and Stroma Cells of Cholangiocarcinoma Patients. *Ann Surg Oncol* 2020; **27**: 599-607 [PMID: 31407173 DOI: 10.1245/s10434-019-07701-4]

39   **Kim RD**, Chung V, Alese OB, El-Rayes BF, Li D, Al-Toubah TE, Schell MJ, Zhou JM, Mahipal A, Kim BH, Kim DW. A Phase 2 Multi-institutional Study of Nivolumab for Patients With Advanced Refractory Biliary Tract Cancer. *JAMA Oncol* 2020; **6**: 888-894 [PMID: 32352498 DOI: 10.1001/jamaoncol.2020.0930]

40   **Wang D**, Lin J, Yang X, Long J, Bai Y, Mao Y, Sang X, Seery S, Zhao H. Combination regimens with PD-1/PD-L1 immune checkpoint inhibitors for gastrointestinal malignancies. *J Hematol Oncol* 2019; **12**: 42 [PMID: 31014381 DOI: 10.1186/s13045-019-0730-9]

41   **Sharma P**, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* 2015; **161**: 205-214 [PMID: 25860605 DOI: 10.1016/j.cell.2015.03.030]

ORIGINAL ARTICLE

Retrospective Study

Development and validation of a prediction model for moderately severe and severe acute pancreatitis in pregnancy

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Abstract

BACKGROUND
The severity of acute pancreatitis in pregnancy (APIP) is correlated with higher risks of maternal and fetal death.

AIM
To develop a nomogram that could predict moderately severe and severe acute pancreatitis in pregnancy (MSIP).

METHODS
Patients with APIP admitted to West China Hospital between January 2012 and December 2018 were included in this study. They were divided into mild acute pancreatitis in pregnancy (MAIP) and MSIP. Characteristic parameters and laboratory results were collected. The training set and test set were randomly divided at a ratio of 7:3. Least absolute shrinkage and selection operator regression was used to select potential prognostic factors. A nomogram was developed by logistic regression. A random forest model was used to validate the stability of the prediction factors. Receiver operating characteristic curves and calibration curves were used to evaluate the model's predictive performance.

RESULTS
A total of 190 patients were included in this study. A total of 134 patients (70.5%) and 56 patients (29.5%) were classified as having MAIP and MSIP, respectively. Four independent predictors (lactate dehydrogenase, triglyceride, cholesterol, and albumin levels) were identified for MSIP. A nomogram prediction model based on these factors was established. The model had areas under the curve of 0.865 and 0.853 in the training and validation sets, respectively. The calibration curves showed that the nomogram has a good consistency.

CONCLUSION
A nomogram including lactate dehydrogenase, triglyceride, cholesterol, and albumin levels as independent predictors was built with good performance for MSIP prediction.

Key Words: Acute pancreatitis; Prediction model; Pregnancy; Severity; Nomogram; Random forest

Core Tip: The severity of acute pancreatitis in pregnancy (APIP) is correlated with higher risks of maternal and fetal death. Few studies have focused on APIP severity prediction. We identified four predictors developed and established a prediction nomogram model for pregnant patients with moderate and severe acute pancreatitis. This model achieved good concordance indexes and may help guide doctors in the managementof APIP.

INTRODUCTION

Acute pancreatitis (AP) is the most common gastrointestinal disease requiring acute admission to the hospital[1]. The incidence of acute pancreatitis in pregnancy (APIP) varies from 1/10000 to 11.3/10000 [2,3]. Geng *et al*[4] showed that APIP contributes to increased maternal death and fetal loss. Previous studies have shown that the maternal and perinatal mortality rates of APIP are as high as 3.3% and 11.6%-18.7%, respectively[4,5]. According to the revised Atlanta classification, AP was classified as mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe acute pancreatitis (SAP)[6]. MSAP and SAP develop in 20% of AP patients. Although, management strategies such as fluid resuscitation, early enteral nutrition, and organ supportive care are usually performed in the clinical setting, the mortality rate of MSAP and SAP can be as high as 35%, which is significantly higher than that of MAP[7,8]. Furthermore, some studies have shown that APIP severity is significantly associated with a higher risk of maternal and fetal death[5,9]. The first week after AP onset is usually defined as the early phase[6]. It would be useful in clinical management if the severity of APIP could be predicted in the early phase. Currently, several prediction systems, including the Acute Physiology and Chronic Health Evaluation, Ranson score, and Bedside Index for Severity in AP, are usually used for AP patients. However, the sensitivity and specificity of these prediction systems are not high enough, and cumbersome items limit their clinical use[10]. At present, few scoring systems have been designed for patients with APIP[11]. Therefore, this study aimed to develop a simple and useful prediction model to predict moderately severe and severe acute pancreatitis in pregnancy (MSIP).

MATERIALS AND METHODS

Study design and patients

We retrospectively collected the medical records of patients who were diagnosed with AP during pregnancy at West China Hospital from January 2012 to December 2018. Patients meeting the following criteria were excluded: (1) Were readmitted (only included first-time record); (2) Received a cesarean section before admission to West China Hospital; (3) Had a length of more than 7 d from AP onset to admission; (4) Had chronic kidney dysfunction; and (5) Had any missing data of candidate variables. The Ethics Committee of West China Hospital approved the study, and it was conducted according to the Declaration of Helsinki.

Data collection

The following clinical variables were collected: age, etiology (hypertriglyceridemia, gallstones, other), comorbidities (hypertension, diabetes, fatty liver), smoking, drinking, length of time from onset to admission, gestational weeks on admission, trimester of pregnancy on admission, blood infection, length of hospital stay (LOS), fetal death, and maternal hospital mortality. All laboratory variables were tested in the hospital, including hematocrit, platelet, white blood cell (WBC), and neutrophil levels. Laboratory variables were collected within 48 h of admission. The average levels of retested laboratory
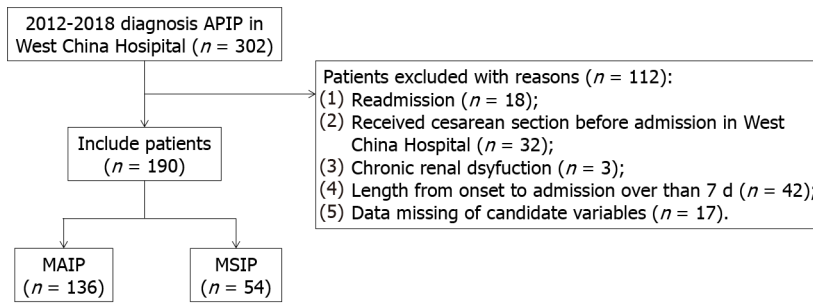
Figure 1 Flow chart of the study. APIP: Acute pancreatitis in pregnancy; MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy.
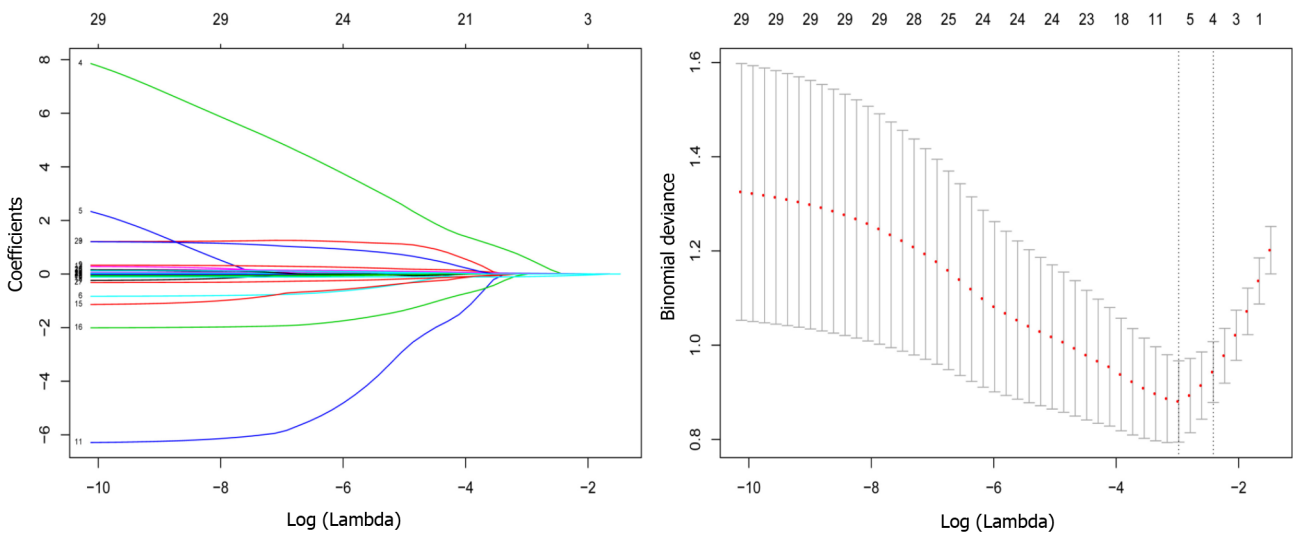

Figure 2 Selection of risk factors of moderately severe and severe acute pancreatitis in pregnancy using the least absolute shrinkage and selection operator logistic regression algorithm. Least absolute shrinkage and selection operator coefficient profiles of the 29 candidate variables. For the optimal lambda, 4 features with a non-0 coefficient were selected.
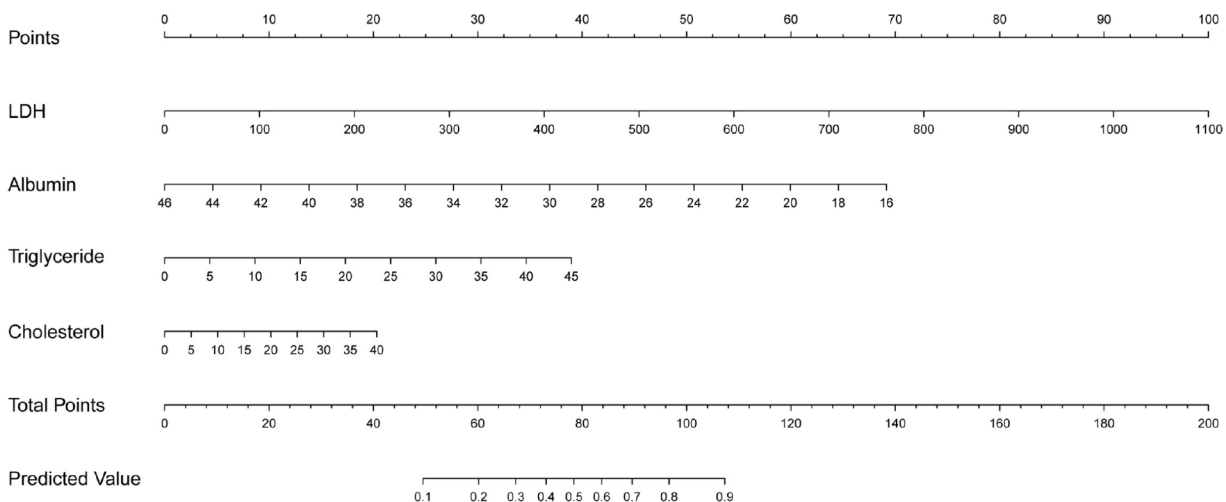
Figure 3 Nomogram for predicting moderately severe and severe acute pancreatitis in pregnancy. Nomogram including four risk factors (lactate dehydrogenase, triglyceride, cholesterol, and albumin were identified as risk factors) to predict moderately severe and severe acute pancreatitis in pregnancy. LDH: Lactate dehydrogenase.

variables are shown.

Candidate variables were age, etiology, comorbidity, smoking, drinking, gestational weeks on admission, trimester of pregnancy on admission, length of time from onset to admission, blood
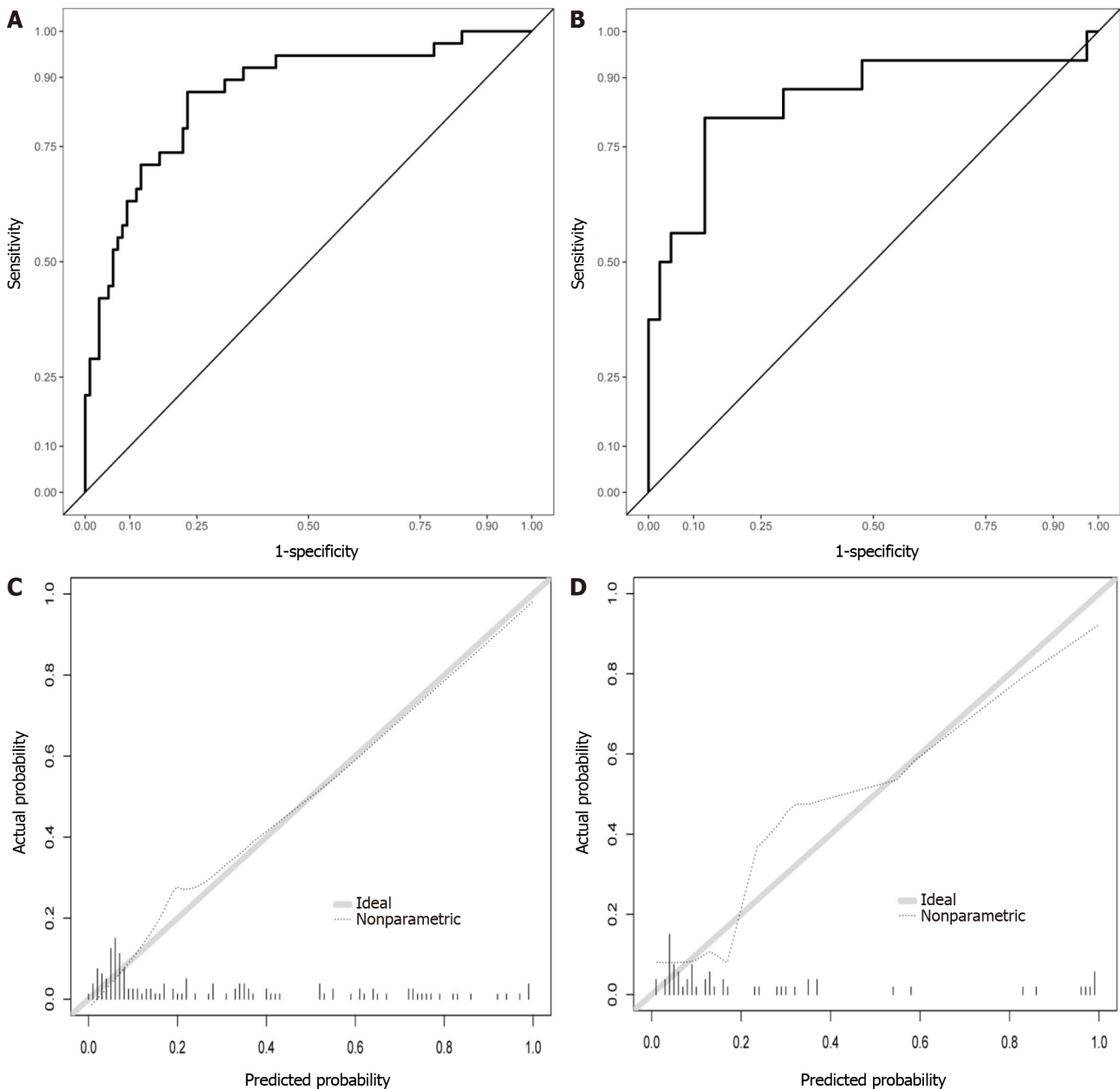
Figure 4 Performance of the nomogram in moderately severe and severe acute pancreatitis in pregnancy prediction. A: Receiver operating characteristic curves in the training set; B: Receiver operating characteristic curves in test set; C: Calibration curves of training set; D: Calibration curves of the test set.

infection, and hematocrit, platelet, WBC, neutrophil, lymphocyte, monocyte, alanine aminotransferase, albumin, creatinine, aspartate aminotransferase, alkaline phosphatase, creatine kinase, lactate dehydrogenase (LDH), triglyceride, cholesterol, high-density lipoprotein, low-density lipoprotein, sodium, potassium, and chlorine levels..

Definitions

According to the revised Atlanta Classification of Acute Pancreatitis[6], a diagnosis of acute pancreatitis requires two of the following three features: (1) abdominal pain consistent with acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) serum lipase activity (or amylase activity) at least three times greater than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on contrast-enhanced computed tomography, and less commonly on magnetic resonance imaging or transabdominal ultrasonography. The grades of severity were also based on the revised Atlanta Classification of Acute Pancreatitis[6]. Patients with persistent organ failure (> 48 h) were classified as having severe acute pancreatitis. Patients with transient organ failure (< 48 h) and/or local or systemic complications without persistent organ failure were classified as having moderately severe acute pancreatitis. Organ failure was classified according to the Modified Marshall scoring system for organ dysfunction[6]. Patients who needed mechanical ventilation or had a
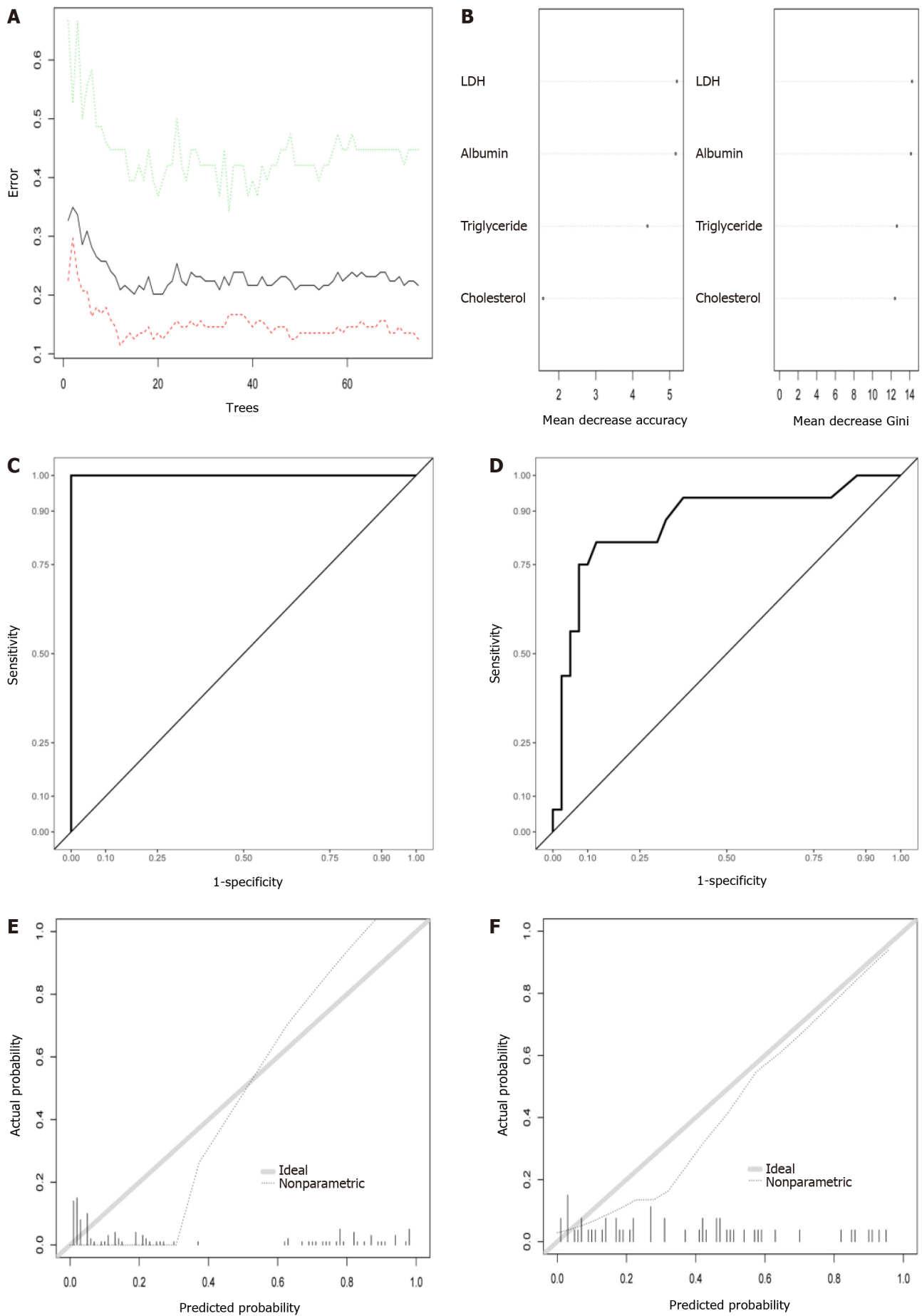
Figure 5 Development and assessment of the random forest algorithm in moderately severe and severe acute pancreatitis in pregnancy

prediction. A: Relationship between out-of-bag error and number of trees. In total, 75 trees are selected to establish a random forest model; B: Feature importance; C: Receiver operating characteristic curves in the training set; D: Receiver operating characteristic curves in test set; E: Calibration curves of training set; F: Calibration curves of the test set.

PaO$_2$/FiO$_2$ ratio less than 300 were diagnosed with respiratory failure. Patient need for vasopressor support was thought to indicate cardiovascular failure. When the serum creatinine level was over 170 μmol/L, renal failure was diagnosed. Blood infection was defined as described in a previous study[12].

Statistical analysis

Data are expressed as the mean ± SD for normally distributed continuous variables and as the median (interquartile range) for nonnormally distributed variables. Categorical data are expressed as numbers (percentages). Student's t-test was used to compare normally distributed continuous variables, and the Wilcoxon rank-sum test was used to compare nonnormally distributed continuous variables. The χ^2-test or Fisher's exact test was used to compare categorical variables. Statistical analysis was performed using R software. (Version 3.6.1) A 2-sided P value < 0.05 was considered statistically significant.

First, least absolute shrinkage and selection operator (LASSO) regression was used to select potential prognostic factors from the candidate variables. Logistic regression was used to develop a nomogram. The random forest model further validated the predictive performance of the selected factors. To reduce the risk of overfitting, the whole dataset was randomly divided into the training set and validation set at a ratio of 7:3. The model's development was based on the training set, and the model's performance assessment was based on the validation set. Finally, a new nomogram based on the selected predictors was established. Receiver operating characteristic (ROC) curves and calibration curves were used to evaluate the model's predictive performance. ROC curves were calculated to estimate the discrimination of the prediction model. Calibration curves were plotted to evaluate the consistency between the predicted MSIP probability and actual MSIP proportion. Values of 1 and 0.5 indicated perfect discrimination and no discrimination, respectively.

RESULTS

Basic characteristics of the participants

Figure 1 shows the flow chart of the study. During the 7 years, 302 patients with APIP were admitted to West China Hospital. A total of 112 patients were excluded for various reasons, such as readmission, having a cesarean section before admission, and missing data. Finally, a total of 190 patients with APIP were included in this study. Among them, 134 patients (70.5%) were classified as having MAIP, and 56 patients (29.5%) were classified as having MSIP. The overall characteristics of the patients are presented in Table 1.

The mean ages of the MAIP and MSIP groups were 27.61 ± 5.25 years and 29.46 ± 5.57 years, respectively. Patients in the MSIP group were significantly older than those in the MAIP group (P = 0.032). The most common cause of APIP in both groups was hypertriglyceridemia. Biliary disease was the second most common cause of APIP, which was found in 45 (33.1%) and 19 (35.2%) patients in the MAIP and MSIP groups, respectively. The number of patients with diabetes in the MSIP group was significantly higher than that in the MAIP group (P = 0.001). The rate of blood infections (P < 0.001) in the MSIP group was significantly higher than that in the MAIP group. The LOS (P < 0.001) in the MSIP group was significantly longer than that in the MAIP group, and the rate of fetal deaths (P < 0.001) in the MSIP group was significantly higher than that in the MAIP group. Other clinical indicators were not different between the two groups.

Laboratory indices such as WBC (P = 0.035), neutrophil (P = 0.019), alanine aminotransferase (P = 0.006), albumin (P < 0.001), creatinine (P < 0.001), alkaline phosphatase (P = 0.020), creatine kinase (P < 0.001), LDH (P < 0.001), triglyceride (P < 0.001), cholesterol (P < 0.001), high density lipoprotein (P = 0.001), and sodium (P = 0.004) levels were significantly different between the two groups (P < 0.05).

Identification and validation of predictive factors for patients with MSIP

Variable selection using the LASSO regression model: The data were randomly divided into the training set and test set at a ratio of 7:3. The characteristics of the patients in the training and test sets are displayed in Table 2. Most of the included variables were well balanced between the two groups. Four variables (albumin, lactate dehydrogenase, triglyceride, and cholesterol levels) had nonzero coefficients in the LASSO regression model based on the analysis of the whole dataset (Figure 2).

Logistic regression development and validation prediction model: Four selected variables albumin, lactate dehydrogenase, triglyceride, and cholesterol levels, were incorporated into the nomogram model (Figure 3). The ROC curves and calibration curves of the training set and test set are shown in Figure 4. The parameters of the ROC curve at the optimal cutoff point are displayed in Table 3. The areas under

Table 1 Demographics and clinical characteristics of acute pancreatitis patients in pregnancy			
Parameters	MAIP (*n* = 136)	MSIP (*n* = 54)	*P* value
Age	27.61 ± 5.25	29.46 ± 5.57	0.032
Etiology			0.514
Hypertriglyceridemia	50 (36.8)	24 (44.4)	
Gallstone	45 (33.1)	19 (35.2)	
Other	41 (30.1)	11 (20.4)	
Comorbidity			
Hypertension	0 (0.0)	2 (3.7)	0.080
Diabetes	8 (5.9)	13 (24.1)	0.001
Fatty liver disease	32 (23.5)	16 (29.6)	0.492
Smoking	3 (2.2)	2 (3.7)	0.937
Drinking	4 (2.9)	1 (1.9)	1.000
Trimester of pregnancy on admission			
Early (1–12 wk)	9 (6.6)	3(5.6)	
Mid (12–24 wk)	31 (22.8)	10(18.5)	
Late (24–40 wk)	96 (70.6)	41(75.9)	
Gestational weeks on admission	28.04 ± 7.72	28.80 ± 6.64	0.520
Onset to admission (days)	1.59 ± 1.37	1.88 ± 1.65	0.220
Blood infection	0 (0.0)	8 (14.8)	< 0.001
LOS	7.25 ± 4.27	11.88 ± 7.42	< 0.001
Fetal death	3 (2.2)	13(24.1)	< 0.001
Maternal hospital mortality	0 (0.0)	1(2.9)	0.284
Hematocrit	0.33 ± 0.05	0.32 ± 0.06	0.155
Platelet	164.42 ± 55.01	147.55 ± 65.17	0.072
WBC	12.59 ± 4.71	14.15 ± 4.15	0.035
Neutrophils	10.86 ± 4.40	12.49 ± 3.97	0.019
Lymphocytes	1.01 ± 0.40	0.88 ± 0.47	0.068
Monocytes	0.55 ± 0.23	0.48 ± 0.27	0.064
Alanine aminotransferase	50.94 ± 78.74	19.57 ± 37.40	0.006
Albumin	34.22 ± 3.70	29.36 ± 5.17	< 0.001
Creatinine	42.57 ± 9.30	75.87 ± 100.15	< 0.001
Aspartate aminotransferase	51.17 ± 67.61	35.07 ± 50.13	0.115
Alkaline phosphatase	113.56 ± 52.19	95.38 ± 36.87	0.020
Creatine kinase	36.20 ± 25.87	126.36 ± 213.49	< 0.001
LDH	185.32 ± 66.39	346.93 ± 208.95	< 0.001
Triglyceride	5.87 ± 6.72	12.57 ± 7.34	< 0.001
Cholesterol	7.34 ± 5.63	12.80 ± 6.64	< 0.001
High density lipoprotein	1.40 ± 0.48	1.16 ± 0.39	0.001
Low density lipoprotein	2.24 ± 1.23	1.94 ± 1.59	0.158
Sodium	135.62 ± 3.82	133.57 ± 5.43	0.004
Potassium	3.76 ± 0.34	3.83 ± 0.46	0.294
Chlorine	102.17 ± 4.46	102.44 ± 6.40	0.735

MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy; LOS: Length of hospital stay; WBC: White blood cell; LDH: Lactate dehydrogenase.

the curve in the training and validation sets were 0.865 and 0.853, respectively. The calibration curves showed that the nomogram has good consistency. The positive predictive value was 0.8750, and the negative predictive value was 0.8125.

Random forest model development and validation prediction model: The relationship between out-of-bag error and the number of trees is shown in Figure 5A. In total, 100 trees were selected to establish a random forest model. Two methods were used to rank the importance of the variables (Figure 5B). The ROC curves are shown in Figure 5C and D, and the optimal cutoff point is displayed in Table 3. In addition, the calibration curves indicated good agreement between the predicted probability and observed probability for MSIP in the training and test sets (Figure 5E and F).

DISCUSSION

APIP was thought to be associated with high rates of maternal death and fetal loss. The early and accurate prediction of APIP severity is of great importance for effective therapy. Previous studies have not only focused on the treatments of APIP[13,14] but have also shown interest in the prediction factors for APIP[15]. A single prediction factor cannot achieve the expected predictive power. Therefore, it is necessary to establish a multifactor model to predict the severity of APIP to help with risk stratification and management. In the present study, a new prediction model consisting of four risk factors (albumin, lactate dehydrogenase, triglyceride, and cholesterol levels) with good predictive value was built and verified.

Hypertriglyceridemia (HTG) induced APIP has received continuous attention[16-18]. HTG-induced AP is defined as AP patients with a triglyceride level above 1000 mg/dL (11.3 mmol/L) alone, or 500 mg/dL (5.65 mmol/L) accompanied by lipemic or lactescent blood, after excluding other etiologies[19]. In a recent study by Olesen *et al*[20], the mean incidence rate of HTG associated pancreatitis was 1.4 (95%CI, 1.1-1.7) per 100000 person-years and it has increased year by year. In addition, AP patients with severe HTG are not rare in Asia[21]. High-fat diets are common among pregnant women in China. In some studies, HTG was the second leading cause of AP in China[22,23]. In our study, HTG (38.9%) was the leading cause of APIP. A higher level of triglycerides not only contributes to more severe pancreatitis[21,24-26] but is also associated with more severe complications[27]. Thus, the detection of HTG is very important in APIP prediction.

As a cytoplasmic enzyme, LDH is widely expressed in tissues. It converts pyruvate to lactate when oxygen is in short supply[28]. In some disease conditions, such as tissue injury, hypoxia, or necrosis, elevated LDH levels are observed[29,30]. As a systemic inflammatory disease, AP can lead to organ dysfunction and pancreatic or peripancreatic necrosis when the disease progresses. Thus, LDH was recognized as a prognostic factor for severe AP in the 1992 Atlanta criteria[31]. More studies have shown that LDH is a useful predictor of AP severity[32,33]. Furthermore, LDH is used not only for the prediction of severity but also for the prediction of organ failure in AP patients[34]. A recent study displayed the high prediction ability of LDH in SAP prediction when levels were over 273.04 U/L[35]. In a study by Cui, an LDH level over 647 U/L showed a good ability to predict persistent organ failure in patients with AP[36]. In this study, LDH was the most important factor in the accuracy and Gini rank of the random forest model. Additionally, LDH accounted for the highest score in the final nomogram model. Moreover, convenient laboratory tests for LDH could be routinely utilized in the clinical setting.

Although hypercholesterolemia is a known risk factor for cardiovascular diseases, with further investigation of AP, the relationship between AP and hypercholesterolemia has been revealed. Hypercholesterolemia may lead to inflammatory responses, lysosomal damage, and proinflammatory cytokine secretion[37,38]. In particular, it promotes the augmentation of toll-like receptor signaling, which plays a significant proinflammatory role in the progression of AP[39]. Clinical studies also found a relationship between cholesterol and AP. Cholesterol is not only associated with AP occurrence[40] but is also thought to be an early predictor of persistent organ failure and mortality in AP patients[41, 42]. Some studies have produced inconsistent conclusions. Some reported that cholesterol was not identified as an independent risk factor for SAP[43,44]. However, cholesterol was thought to be a predictor of SAP development in the study by Hong *et al*[45]. Thus, it is unclear whether the relationship between AP severity and cholesterol is linear. A recent study suggested that cholesterol levels have a U-shaped association with AP severity[46]. This may explain the different conclusions in previous studies.

Some studies have shown that decreases in albumin levels predict the severity of AP[47,48]. An albumin level less than 30 g/L was an independent risk factor for acute respiratory distress syndrome in SAP patients[49]. In the present study, the albumin levels of patients in the MSIP group were less than

Table 2 Demographic and clinical characteristics of patients in training group

Parameters	Training set			Test set		
	MAIP (96)	MSIP (38)	*P* value	MAIP (40)	MSIP (16)	*P* value
Age	27.16 ± 5.46	30.13 ± 6.09	0.007	28.70 ± 4.60	27.88 ± 3.79	0.528
Etiology			0.620			0.804
Hypertriglyceridemia	32 (33.3)	18 (47.4)		18 (45.0)	7 (43.8)	
Gallstone	36 (37.5)	13 (34.2)		9 (22.5)	5 (31.2)	
Other	28 (29.2)	7 (18.4)		13 (32.5)	4 (25.0)	
Comorbidity						
Hypertension	0 (0.0)	2 (5.3)	0.079	0 (0.0)	0 (0.0)	-
Diabetes	6 (6.3)	9 (23.7)	0.012	2 (5.0)	4 (25.0)	0.049
Fatty liver disease	20 (20.8)	11 (28.9)	0.437	12 (30.0)	5 (31.2)	1.000
Smoking	1 (1.0)	2 (5.3)	0.400	2 (5.0)	0 (0.0)	0.909
Drinking	3 (3.1)	1 (2.6)	1.000	1 (2.5)	0 (0.0)	1.000
Trimester of pregnancy on admission						
Early (1–12 wk)	6 (6.3)	2 (5.3)		3 (7.5)	1 (6.3)	
Mid (12–24 wk)	22 (22.9)	8 (21.1)		9 (22.5)	2 (12.5)	
Late (24–40 wk)	68 (70.8)	28 (73.7)		28 (70.0)	13 (81.3)	
Gestational weeks on admission	27.53 ± 7.52	29.74 ± 6.34	0.113	29.25 ± 8.15	26.56 ± 7.01	0.252
Onset to admission (d)	1.63 ± 1.35	2.12 ± 1.87	0.090	1.50 ± 1.44	1.29 ± 0.63	0.586
Blood infection	0 (0.0)	6 (15.8)	< 0.001	0 (0.0)	2 (12.5)	0.139
LOS	6.99 ± 4.43	23.11 ± 48.52	< 0.001	7.90 ± 3.63	18.25 ± 12.96	0.001
Fetal death	1 (1.0)	9 (23.7)	< 0.001	2 (5.0)	4 (25.0)	0.049
Maternal hospital mortality	0 (0.0)	1 (2.6)	0.284	0 (0.0)	0 (0.0)	-
Hematocrit	0.33 ± 0.05	0.32 ± 0.06	0.734	0.33 ± 0.05	0.30 ± 0.05	0.040
Platelet	169.57 ± 56.91	141.97 ± 61.66	0.015	152.07 ± 48.61	160.80 ± 73.24	0.604
WBC	12.89 ± 4.82	13.94 ± 4.10	0.239	11.87 ± 4.42	14.64 ± 4.37	0.038
Neutrophils	11.19 ± 4.52	12.34 ± 3.83	0.166	10.09 ± 4.06	12.85 ± 4.39	0.029
Lymphocytes	1.00 ± 0.38	0.91 ± 0.49	0.246	1.01 ± 0.44	0.81 ± 0.41	0.129
Monocytes	0.55 ± 0.23	0.48 ± 0.29	0.147	0.56 ± 0.25	0.47 ± 0.22	0.249
Alanine aminotransferase	55.20 ± 82.18	22.91 ± 44.14	0.024	40.73 ± 69.69	11.62 ± 6.40	0.103
Albumin	34.44 ± 3.72	29.68 ± 5.49	< 0.001	33.71 ± 3.62	28.62 ± 4.41	< 0.001
Creatinine	42.90 ± 9.16	86.27 ± 117.45	< 0.001	41.78 ± 9.69	51.17 ± 22.10	0.030
Aspartate aminotransferase	54.45 ± 69.25	40.67 ± 58.67	0.281	43.33 ± 63.66	21.77 ± 10.97	0.186
Alkaline phosphatase	114.05 ± 51.61	100.65 ± 37.29	0.148	112.39 ± 54.23	82.86 ± 33.70	0.048
creatine kinase	36.46 ± 26.55	129.89 ± 242.89	< 0.001	35.58 ± 24.47	117.98 ± 124.10	< 0.001
LDH	183.85 ± 63.85	356.97 ± 234.19	< 0.001	188.86 ± 72.86	323.09 ± 134.59	< 0.001
Triglyceride	5.44 ± 6.86	12.62 ± 8.01	< 0.001	6.91 ± 6.33	12.46 ± 5.66	0.004
cholesterol	6.79 ± 4.43	12.19 ± 6.18	< 0.001	8.68 ± 7.70	14.24 ± 7.63	0.018
High density lipoprotein	1.42 ± 0.49	1.18 ± 0.39	0.007	1.34 ± 0.43	1.12 ± 0.40	0.078
Low density lipoprotein	2.27 ± 1.18	2.00 ± 1.58	0.282	2.17 ± 1.35	1.78 ± 1.67	0.364
Sodium	135.91 ± 3.19	133.94 ± 5.71	0.012	134.93 ± 5.01	132.68 ± 4.72	0.129
Potassium	3.76 ± 0.30	3.88 ± 0.47	0.075	3.78 ± 0.44	3.71 ± 0.45	0.588

Chlorine	102.63 ± 4.22	103.14 ± 6.80	0.597	101.06 ± 4.85	100.78 ± 5.14	0.849

MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy; LOS: Length of hospital stay; WBC: White blood cell; LDH: Lactate dehydrogenase.

Table 3 Receiver operating characteristic curves at the optimal cut-off point according to different models			
Models	AUC	Sensitivity	Specificity
Training set			
Logistic model	0.865	0.868	0.771
Random forest model	1.000	1.000	1.000
Validation set			
Logistic model	0.853	0.812	0.875
Random forest model	0.870	0.812	0.875

AUC: Area under the receiver operating characteristic curve.

30 g/L and significantly lower than those of patients in the MAIP group. This was in accordance with previous studies.

Lactate dehydrogenase, triglyceride, albumin, and cholesterol are routine test items in clinical practice. They can be easily detected from blood samples at a low cost. Therefore, this nomogram will be easy to use and function for MSIP prediction in the clinical setting.

There are some limitations to this study. First, the sample size of 190 patients with APIP was greater than those of most previous studies, but the sample size of this study was still small. Second, this was a retrospective study, so some data were missing. Thus, some variables were not included in this study. Third, the prediction model has a good prediction ability of MSIP (consisting of MSAP and SAP), but further differentiation of MSAP and SAP cannot be achieved. The prognosis of MSAP is not as poor as that of SAP. Thus, separate predictions of MSAP and SAP should be considered in future studies. Moreover, this study only collected data from our institution. If validation can be performed in external institutions, the conclusion of this study would be more substantial.

CONCLUSION

We developed and validated a nomogram with good accordance for the prediction of MSIP. Incorporating blood indices for albumin, lactate dehydrogenase, triglyceride, and cholesterol levels into the nomogram facilitates the early individualized prediction of APIP severity.

ARTICLE HIGHLIGHTS

Research background
The severity of acute pancreatitis in pregnancy is correlated with higher risks of maternal and fetal death.

Research motivation
There is a lack of a scoring model for predicting the moderately severe and severe acute pancreatitis in pregnancy (MSIP).

Research objectives
We aimed to develop a prediction model for moderately severe and severe acute pancreatitis in pregnancy.

Research methods
The training set and test set were randomly divided at a ratio of 7:3. Least absolute shrinkage and selection operator regression was used to select potential prognostic factors. A nomogram was developed by logistic regression. A random forest model was used to validate the stability of the of

prediction factors. Receiver operating characteristic curves and calibration curves were used to evaluate the model's predictive performance.

Research results

A total of 190 patients were included in this study. Four predictors including lactate dehydrogenase, triglyceride, cholesterol, and albumin levels constitute the prediction model. The model had areas under the curve of 0.865 and 0.853 in the training and validation sets, respectively. The calibration curves showed that the prediction model has a good consistency.

Research conclusions

An effective prediction model that can predict MSIP was constructed.

Research perspectives

Our model could help to predict moderately severe and severe acute pancreatitis in pregnancy. Usability of the model needs validation by other center data.

FOOTNOTES

REFERENCES

1   **Boxhoorn L**, Voermans RP, Bouwense SA, Bruno MJ, Verdonk RC, Boermeester MA, van Santvoort HC, Besselink MG. Acute pancreatitis. *Lancet* 2020; **396**: 726-734 [PMID: 32891214 DOI: 10.1016/S0140-6736(20)31310-6]

2   **Magudapathi C**, Shanthi S, Palanisamy R. Pancreatitis in Pregnancy: Case Series for 5 Years. *J Obstet Gynaecol India* 2020; **70**: 169-172 [PMID: 32255957 DOI: 10.1007/s13224-019-01267-7]

3   **Tang SJ**, Rodriguez-Frias E, Singh S, Mayo MJ, Jazrawi SF, Sreenarasimhaiah J, Lara LF, Rockey DC. Acute pancreatitis during pregnancy. *Clin Gastroenterol Hepatol* 2010; **8**: 85-90 [PMID: 19747985 DOI: 10.1016/j.cgh.2009.08.035]

4   **Geng Y**, Li W, Sun L, Tong Z, Li N, Li J. Severe acute pancreatitis during pregnancy: eleven years experience from a surgical intensive care unit. *Dig Dis Sci* 2011; **56**: 3672-3677 [PMID: 21735079 DOI: 10.1007/s10620-011-1809-5]

5   **Luo L**, Zen H, Xu H, Zhu Y, Liu P, Xia L, He W, Lv N. Clinical characteristics of acute pancreatitis in pregnancy: experience based on 121 cases. *Arch Gynecol Obstet* 2018; **297**: 333-339 [PMID: 29164335 DOI: 10.1007/s00404-017-4558-7]

6   **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

7   **van Dijk SM**, Hallensleben NDL, van Santvoort HC, Fockens P, van Goor H, Bruno MJ, Besselink MG; Dutch Pancreatitis Study Group. Acute pancreatitis: recent advances through randomised trials. *Gut* 2017; **66**: 2024-2032 [PMID: 28838972 DOI: 10.1136/gutjnl-2016-313595]

8   **Lankisch PG**, Apte M, Banks PA. Acute pancreatitis. *Lancet* 2015; **386**: 85-96 [PMID: 25616312 DOI: 10.1016/S0140-6736(14)60649-8]

9   **Sun L**, Li W, Geng Y, Shen B, Li J. Acute pancreatitis in pregnancy. *Acta Obstet Gynecol Scand* 2011; **90**: 671-676 [PMID: 21306332 DOI: 10.1111/j.1600-0412.2011.01072.x]

10   **Cho JH**, Kim TN, Chung HH, Kim KH. Comparison of scoring systems in predicting the severity of acute pancreatitis. *World J Gastroenterol* 2015; **21**: 2387-2394 [PMID: 25741146 DOI: 10.3748/wjg.v21.i8.2387]

11   **Yang Z**, Guo G, Li H. Predicting fetal loss in severe acute pancreatitis during pregnancy: a 5-year single-tertiary-center retrospective analysis. *Postgrad Med* 2020; **132**: 473-478 [PMID: 32249649 DOI: 10.1080/00325481.2020.1752010]

12   **Huerta LE**, Rice TW. Pathologic Difference between Sepsis and Bloodstream Infections. *J Appl Lab Med* 2019; **3**: 654-663 [PMID: 31639733 DOI: 10.1373/jalm.2018.026245]

13   **Goto S**, Ookawara S, Tabei K. Effectiveness of Plasma Exchange for Acute Pancreatitis Induced by Hypertriglyceridemia During Pregnancy. *Ther Apher Dial* 2016; **20**: 98-99 [PMID: 26626304 DOI: 10.1111/1744-9987.12373]

14   **Talebi-Bakhshayesh M**, Mohammadzadeh A, Zargar A. Timing of cholecystectomy after acute severe pancreatitis in pregnancy. *Malays J Med Sci* 2015; **22**: 68-70 [PMID: 26715899]

15   **Zhang L**, Wang Y, Han J, Shen H, Zhao M, Cai S. Neutrophil-lymphocyte ratio, gamma-glutamyl transpeptidase, lipase, high-density lipoprotein as a panel of factors to predict acute pancreatitis in pregnancy. *Medicine (Baltimore)* 2018; **97**: e11189 [PMID: 29952970 DOI: 10.1097/MD.0000000000011189]

16   **Tan SYT**, Teh SP, Kaushik M, Yong TT, Durai S, Tien CJ, Gardner DS. Hypertriglyceridemia-induced pancreatitis in pregnancy: case review on the role of therapeutic plasma exchange. *Endocrinol Diabetes Metab Case Rep* 2021; **2021** [PMID: 34013888 DOI: 10.1530/EDM-21-0017]

17   **Zeng L**, Cai X, Chen J, Jin G, Zheng Y. Role of mean platelet volume in hypertriglyceridemia-induced acute pancreatitis during pregnancy. *BMC Pregnancy Childbirth* 2020; **20**: 592 [PMID: 33023512 DOI: 10.1186/s12884-020-03295-y]

18   **Chyzhyk V**, Kozmic S, Brown AS, Hudgins LC, Starc TJ, Davila AD, Blevins TC, Diffenderfer MR, He L, Geller AS, Rush C, Hegele RA, Schaefer EJ. Extreme hypertriglyceridemia: Genetic diversity, pancreatitis, pregnancy, and prevalence. *J Clin Lipidol* 2019; **13**: 89-99 [PMID: 30352774 DOI: 10.1016/j.jacl.2018.09.007]

19   **Zafrir B**, Jubran A, Hijazi R, Shapira C. Clinical features and outcomes of severe, very severe, and extreme hypertriglyceridemia in a regional health service. *J Clin Lipidol* 2018; **12**: 928-936 [PMID: 29685592 DOI: 10.1016/j.jacl.2018.03.086]

20   **Olesen SS**, Harakow A, Krogh K, Drewes AM, Handberg A, Christensen PA. Hypertriglyceridemia is often under recognized as an aetiologic risk factor for acute pancreatitis: A population-based cohort study. *Pancreatology* 2021; **21**: 334-341 [PMID: 33608229 DOI: 10.1016/j.pan.2021.02.005]

21   **Jo SI**, Chang JH, Kim TH, Kim CW, Kim JK, Han SW. Subsets associated with developing acute pancreatitis in patients with severe hypertriglyceridemia and the severity of pancreatitis. *Pancreatology* 2019; **19**: 795-800 [PMID: 31421975 DOI: 10.1016/j.pan.2019.08.002]

22   **Yin G**, Cang X, Yu G, Hu G, Ni J, Xiong J, Hu Y, Xing M, Chen C, Huang Y, Tang M, Zhao Y, Cheng G, Wan R, Wang S, Wang X. Different Clinical Presentations of Hyperlipidemic Acute Pancreatitis: A Retrospective Study. *Pancreas* 2015; **44**: 1105-1110 [PMID: 26348469 DOI: 10.1097/MPA.0000000000000403]

23   **Jin M**, Bai X, Chen X, Zhang H, Lu B, Li Y, Lai Y, Qian J, Yang H. A 16-year trend of etiology in acute pancreatitis: The increasing proportion of hypertriglyceridemia-associated acute pancreatitis and its adverse effect on prognosis. *J Clin Lipidol* 2019; **13**: 947-953.e1 [PMID: 31735687 DOI: 10.1016/j.jacl.2019.09.005]

24   **Valdivielso P**, Ramírez-Bueno A, Ewald N. Current knowledge of hypertriglyceridemic pancreatitis. *Eur J Intern Med* 2014; **25**: 689-694 [PMID: 25269432 DOI: 10.1016/j.ejim.2014.08.008]

25   **Vipperla K**, Somerville C, Furlan A, Koutroumpakis E, Saul M, Chennat J, Rabinovitz M, Whitcomb DC, Slivka A, Papachristou GI, Yadav D. Clinical Profile and Natural Course in a Large Cohort of Patients With Hypertriglyceridemia and Pancreatitis. *J Clin Gastroenterol* 2017; **51**: 77-85 [PMID: 27322530 DOI: 10.1097/MCG.0000000000000579]

26   **Wang SH**, Chou YC, Shangkuan WC, Wei KY, Pan YH, Lin HC. Relationship between Plasma Triglyceride Level and Severity of Hypertriglyceridemic Pancreatitis. *PLoS One* 2016; **11**: e0163984 [PMID: 27727299 DOI: 10.1371/journal.pone.0163984]

27   **Deng LH**, Xue P, Xia Q, Yang XN, Wan MH. Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis. *World J Gastroenterol* 2008; **14**: 4558-4561 [PMID: 18680239 DOI: 10.3748/wjg.14.4558]

28   **Feron O**. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. *Radiother Oncol* 2009; **92**: 329-333 [PMID: 19604589 DOI: 10.1016/j.radonc.2009.06.025]

29   **Karlsson M**, Wiberg-Itzel E, Chakkarapani E, Blennow M, Winbladh B, Thoresen M. Lactate dehydrogenase predicts hypoxic ischaemic encephalopathy in newborn infants: a preliminary study. *Acta Paediatr* 2010; **99**: 1139-1144 [PMID: 20236255 DOI: 10.1111/j.1651-2227.2010.01802.x]

30   **Kato GJ**, McGowan V, Machado RF, Little JA, Taylor J 6th, Morris CR, Nichols JS, Wang X, Poljakovic M, Morris SM Jr, Gladwin MT. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood* 2006; **107**: 2279-2285 [PMID: 16291595 DOI: 10.1182/blood-2005-06-2373]

31   **Chen CC**, Wang SS, Chao Y, Lu CW, Lee SD, Tsai YT, Lo KJ. C-reactive protein and lactate dehydrogenase isoenzymes in the assessment of the prognosis of acute pancreatitis. *J Gastroenterol Hepatol* 1992; **7**: 363-366 [PMID: 1515559 DOI: 10.1111/j.1440-1746.1992.tb00998.x]

32   **Losurdo G**, Iannone A, Principi M, Barone M, Ranaldo N, Ierardi E, Di Leo A. Acute pancreatitis in elderly patients: A

retrospective evaluation at hospital admission. *Eur J Intern Med* 2016; **30**: 88-93 [PMID: 26806437 DOI: 10.1016/j.ejim.2016.01.011]

33   **Ćeranić DB**, Zorman M, Skok P. Interleukins and inflammatory markers are useful in predicting the severity of acute pancreatitis. *Bosn J Basic Med Sci* 2020; **20**: 99-105 [PMID: 31242405 DOI: 10.17305/bjbms.2019.4253]

34   **Cui J**, Xiong J, Zhang Y, Peng T, Huang M, Lin Y, Guo Y, Wu H, Wang C. Serum lactate dehydrogenase is predictive of persistent organ failure in acute pancreatitis. *J Crit Care* 2017; **41**: 161-165 [PMID: 28554094 DOI: 10.1016/j.jcrc.2017.05.001]

35   **Tian F**, Li H, Wang L, Li B, Aibibula M, Zhao H, Feng N, Lv J, Zhang G, Ma X. The diagnostic value of serum C-reactive protein, procalcitonin, interleukin-6 and lactate dehydrogenase in patients with severe acute pancreatitis. *Clin Chim Acta* 2020; **510**: 665-670 [PMID: 32828732 DOI: 10.1016/j.cca.2020.08.029]

36   **Plesko M**, Suvada J, Makohusova M, Waczulikova I, Behulova D, Vasilenkova A, Vargova M, Stecova A, Kaiserova E, Kolenova A. The role of CRP, PCT, IL-6 and presepsin in early diagnosis of bacterial infectious complications in paediatric haemato-oncological patients. *Neoplasma* 2016; **63**: 752-760 [PMID: 27468879 DOI: 10.4149/neo_2016_512]

37   **Triantafilou M**, Miyake K, Golenbock DT, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. *J Cell Sci* 2002; **115**: 2603-2211 [PMID: 12045230 DOI: 10.1242/jcs.115.12.2603]

38   **Li HB**, Jin C, Chen Y, Flavell RA. Inflammasome activation and metabolic disease progression. *Cytokine Growth Factor Rev* 2014; **25**: 699-706 [PMID: 25156419 DOI: 10.1016/j.cytogfr.2014.07.020]

39   **Sharif R**, Dawra R, Wasiluk K, Phillips P, Dudeja V, Kurt-Jones E, Finberg R, Saluja A. Impact of toll-like receptor 4 on the severity of acute pancreatitis and pancreatitis-associated lung injury in mice. *Gut* 2009; **58**: 813-819 [PMID: 19201771 DOI: 10.1136/gut.2008.170423]

40   **Shen Z**, Wang X, Zhen Z, Wang Y, Sun P. Metabolic syndrome components and acute pancreatitis: a case-control study in China. *BMC Gastroenterol* 2021; **21**: 17 [PMID: 33407178 DOI: 10.1186/s12876-020-01579-3]

41   **Zhou CL**, Zhang CH, Zhao XY, Chen SH, Liang HJ, Hu CL, Chen NW. Early prediction of persistent organ failure by serum apolipoprotein A-I and high-density lipoprotein cholesterol in patients with acute pancreatitis. *Clin Chim Acta* 2018; **476**: 139-145 [PMID: 29183667 DOI: 10.1016/j.cca.2017.11.028]

42   **Zhang Y**, Guo F, Li S, Wang F, Meng Z, Zhao J, Liu Z, Wang B, Fan P, Wang C, Wu H. Decreased high density lipoprotein cholesterol is an independent predictor for persistent organ failure, pancreatic necrosis and mortality in acute pancreatitis. *Sci Rep* 2017; **7**: 8064 [PMID: 28808236 DOI: 10.1038/s41598-017-06618-w]

43   **Peng YS**, Chen YC, Tian YC, Yang CW, Lien JM, Fang JT, Wu CS, Hung CF, Hwang TL, Tsai YH, Lee MS, Tsai MH. Serum levels of apolipoprotein A-I and high-density lipoprotein can predict organ failure in acute pancreatitis. *Crit Care* 2015; **19**: 88 [PMID: 25851781 DOI: 10.1186/s13054-015-0832-x]

44   **Khan J**, Nordback I, Sand J. Serum lipid levels are associated with the severity of acute pancreatitis. *Digestion* 2013; **87**: 223-228 [PMID: 23751273 DOI: 10.1159/000348438]

45   **Hong W**, Lin S, Zippi M, Geng W, Stock S, Zimmer V, Xu C, Zhou M. High-Density Lipoprotein Cholesterol, Blood Urea Nitrogen, and Serum Creatinine Can Predict Severe Acute Pancreatitis. *Biomed Res Int* 2017; **2017**: 1648385 [PMID: 28904946 DOI: 10.1155/2017/1648385]

46   **Hong W**, Zimmer V, Basharat Z, Zippi M, Stock S, Geng W, Bao X, Dong J, Pan J, Zhou M. Association of total cholesterol with severe acute pancreatitis: A U-shaped relationship. *Clin Nutr* 2020; **39**: 250-257 [PMID: 30772093 DOI: 10.1016/j.clnu.2019.01.022]

47   **Farrell PR**, Hornung L, Farmer P, DesPain AW, Kim E, Pearman R, Neway B, Serrette A, Sehgal S, Heubi JE, Lin TK, Nathan JD, Vitale DS, Abu-El-Haija M. Who's at Risk? *J Pediatr Gastroenterol Nutr* 2020; **71**: 536-542 [PMID: 32541203 DOI: 10.1097/MPG.0000000000002807]

48   **Li S**, Zhang Y, Li M, Xie C, Wu H. Serum albumin, a good indicator of persistent organ failure in acute pancreatitis. *BMC Gastroentero.* 2017; **17**: 59 [PMID: 28446147 DOI: 10.1186/s12876-017-0615-8]

49   **Zhang W**, Zhang M, Kuang Z, Huang Z, Gao L, Zhu J. The risk factors for acute respiratory distress syndrome in patients with severe acute pancreatitis: A retrospective analysis. *Medicine (Baltimore)* 2021; **100**: e23982 [PMID: 33466140 DOI: 10.1097/MD.0000000000023982]

LETTER TO THE EDITOR

Role of magnifying narrow-band imaging endoscopy for diagnosis of *Helicobacter pylori* infection and gastric precancerous conditions: Few issues

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Abstract

Standard endoscopy with biopsy and narrow-band imaging with guided biopsy are techniques for the detection of *Helicobacter pylori (H. pylori)*-related gastritis and precancerous lesions. In this study, the authors compared standard endoscopy and magnified narrow-band imaging (commonly known as NBI-M) in the diagnosis of *H. pylori* infections, atrophic gastritis, and intestinal metaplasia. Although the sensitivity of NBI-M is better than standard endoscopy, the diagnostic accuracy did not differ substantially between the diagnostic modalities. Future prospective studies may guide endoscopists in difficult cases regarding which modality is more useful and cost-effective for the diagnosis of *H. pylori*-related gastritis and precancerous conditions.

Key Words: Standard endoscopy; Magnified narrow band imaging; *Helicobacter pylori*; Atrophic gastritis; Intestinal metaplasia; Pepsinogen

Core Tip: Magnified narrow band imaging (NBI-M) is used for diagnosis of dysplastic and cancerous lesions. The study is the first of its kind to evaluate this modality for detection of *Helicobacter pylori* related gastritis and precancerous lesions. A procedure to be widely accepted should be cost effective and less time consuming. Whereas white light endoscopy is commonly used by endoscopist to detect any cancer or precancerous lesions, formal endoscopic training regarding use of NBI-M enhances feasibility and detection rate. Whether the combination of NBI-M and artificial intelligence can replace biopsy remains a million dollar question.

TO THE EDITOR

We read with avid interest the study by Cho *et al*[1]. The authors compared standard endoscopy and magnified narrow-band imaging (NBI-M) in the diagnosis of *Helicobacter pylori* (*H. pylori*) infections, atrophic gastritis (AG) and intestinal metaplasia (IM). The authors have done excellent work comparing the role of NBI-M and standard endoscopy in the diagnosis of these entities.

Although several studies show the benefits of NBI-M in the diagnosis and characterization of AG and IM, this is perhaps one of the first studies that evaluated the role of NBI-M in *H. pylori* infections[2,3]. We want to raise a few minor points for discussion.

The financial implications and procedure time of NBI-M in relation to standard endoscopy should have been compared. The authors discussed that routine normal white light endoscopy combined with mucosal biopsies is time-consuming and costly. However, we feel that NBI-M evaluation for *H. pylori* infections, IM, and AG may increase procedure time (especially for an inexperienced endoscopist) and have an additional economic impact on the patients. The present study showed that there is no substantial disparity between standard endoscopy and NBI-M with respect to the diagnostic accuracy for detection of *H. pylori* gastritis, severe atrophy, and IM. The economic and time aspect will decide the utility of this procedure. Hence, future prospective studies should focus on this. Moreover, diagnostic accuracy was demonstrably low for the detection of precancerous lesions (72.6% and 61.1% for severe atrophy and IM in the corpus, respectively) even with NBI-M.

The experience of the endoscopist plays a significant role when the question of a definitive diagnosis of *H. pylori* gastritis and precancerous lesions using NBI arises. The diagnostic probability of AG and IM increases when the endoscopist has formal training regarding the diagnosis of AG and IM[4]. The authors have rightly pointed out this limitation in their study that a single experienced endoscopist was used. Artificial intelligence with NBI-M will likely increase the diagnostic yield. However, future robust prospective studies are required to confirm this hypothesis.

FOOTNOTES

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REFERENCES

1   **Cho JH**, Jeon SR, Jin SY, Park S. Standard *vs* magnifying narrow-band imaging endoscopy for diagnosis of *Helicobacter pylori* infection and gastric precancerous conditions. *World J Gastroenterol* 2021; **27**: 2238-2250 [PMID: 34025076 DOI: 10.3748/wjg.v27.i18.2238]

2   **Uedo N**, Ishihara R, Iishi H, Yamamoto S, Yamada T, Imanaka K, Takeuchi Y, Higashino K, Ishiguro S, Tatsuta M. A new method of diagnosing gastric intestinal metaplasia: narrow-band imaging with magnifying endoscopy. *Endoscopy* 2006; **38**: 819-824 [PMID: 17001572 DOI: 10.1055/s-2006-944632]

3   **Savarino E**, Corbo M, Dulbecco P, Gemignani L, Giambruno E, Mastracci L, Grillo F, Savarino V. Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia. *World J Gastroenterol* 2013; **19**: 2668-2675 [PMID: 23674874 DOI: 10.3748/wjg.v19.i17.2668]

4   **Uedo N**, Fujishiro M, Goda K, Hirasawa D, Kawahara Y, Lee JH, Miyahara R, Morita Y, Singh R, Takeuchi M, Wang S, Yao T. Role of narrow band imaging for diagnosis of early-stage esophagogastric cancer: current consensus of experienced endoscopists in Asia-Pacific region. *Dig Endosc* 2011; **23** Suppl 1: 58-71 [PMID: 21535204 DOI: 10.1111/j.1443-1661.2011.01119.x]

Therapeutic drug monitoring in inflammatory bowel disease treatments

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Abstract

Recently, biological drugs have played a leading role in the treatment of inflammatory bowel disease, and therapeutic drug monitoring (TDM) may be useful in maximizing their effectiveness. TDM involves the measurement of serum drug and anti-drug antibodies concentrations as the basis for dosage adjustments or drug conversions to achieve a higher response rate. We believe that concentration thresholds should be individualized based on patients' disease severity, extent and phenotype, and therapeutic purposes should also be considered, with higher cut-offs mainly needed for endoscopic and fistula healing than for symptomatic remission. Proactive and reactive TDM can help optimize treatment, especially in patients receiving anti-tumour necrosis factor, and guide dose adjustment or drug conversion with lower cost. TDM is a promising approach to achieve precision medicine and targeted medicine in the future.

Key Words: Therapeutic drug monitoring; Inflammatory bowel disease; Biologic therapies; Reactive; Proactive; Cost-effective

Core Tip: Therapeutic drug monitoring (TDM) has proven to be useful in the management of patients with inflammatory bowel disease (IBD). The therapeutic value, feasibility and application prospect of TDM in the treatment of IBD were discussed.

TO THE EDITOR

We read with interest the review by Albader *et al*[1] on the application of therapeutic drug monitoring (TDM) in patients with inflammatory bowel disease (IBD). The authors provided a comprehensive overview of the relationship between proactive or reactive TDM and clinical outcomes.

The treatment of IBD has progressed from the original mesalamine to glucocorticoids and immunosuppressants to biologics. Currently, biologic therapy is required by many patients to achieve and maintain clinical and endoscopic remission. However, up to one-third of patients receiving this treatment are primary non-responders, and some patients who show an initial response can also lose response over time[2]. TDM is a useful tool for managing patients on biologic therapy, especially those receiving anti-tumor necrosis factor (anti-TNF) therapy, and it can be used to monitor dose escalation, de-escalation or drug conversion by measuring serum drug concentrations and anti-drug antibodies (ADAs).

However, there does not seem to be a universal optimal cut-off for drug serum concentrations, and the majority of studies have shown that higher serum concentrations are associated with an increased likelihood of clinical response. In 2021, a prospective study of 32 pediatric patients demonstrated that children who achieved endoscopic remission at six months had significantly higher infliximab (IFX) concentrations at different time points during induction (at weeks 4, 6, and 12 as the start of maintenance therapy), and the IFX concentration ≥ 5.0 μg/mL at week 12 was a minimal target to achieve endoscopic remission at six months (area under the receiver operating characteristic curve: 0.796)[3]. A retrospective observational case–control study found that IFX levels below 6.8 μg/mL and antibodies to IFX levels above 4.3 μg/mL before the second infusion were associated with primary nonresponse, especially among patients with Crohn's disease (CD)[4]. A prospective observational study by Kennedy *et al*[5] showed that in multivariable analysis, the only factor independently associated with primary nonresponse was low drug concentration at week 14 [IFX: OR 0.35, P = 0.00038; adalimumab (ADA): OR 0.13, P < 0.0001], the optimal week 14 drug concentrations associated with remission at both week 14 and week 54 were 7 mg/L for IFX and 12 mg/L for ADA. Importantly, we believe that concentration thresholds should be individualized based on patients' disease severity, extent and phenotype, and therapeutic purposes should also be considered, with higher cut-offs mainly needed for endoscopic and fistula healing than for symptomatic remission.

TDM has not been widely recommended for non-anti-TNF biologicals. A study published in 2018 concluded that potential target vedolizumab (VDZ) concentrations at weeks 6 and 14 and steady state during treatment were proposed to be > 37.1, > 18.4 and > 12.7 μg/mL, respectively[6]. There are no definitive conclusions to guide practitioners regarding the target VDZ concentration for achieving endoscopic remission. A review published in 2020 noted that data from registration trials and real-world cohorts suggested an exposure-efficacy relationship of VDZ in patients with IBD, but future studies need to define an upper limit beyond which dose optimization is very unlikely to further increase response rates[7]. Ustekinumab (UST) is a monoclonal antibody against IgG that affects the immunity of IBD patients by binding to the P40 subunit common to interleukin 12 and interleukin 23. According to a review published in 2021, serum UST concentrations are associated with clinical, biochemical, and histological remissions in most clinical trials[8]. A multicenter crosssectional observational trial based on 110 CD patients concluded that there was no association between short-term clinical outcomes and UST concentrations[9]. We can assume that there is an exposure-efficacy association with UST based on current studies. Further study is required to identify the threshold below which dose optimization may be useful. The Janus kinase inhibitor tofacitinib is not impacted by enzyme polymorphisms or disease activity and is not expected to stimulate the formation of neutralizing ADAs. In addition, the drug concentration is not a meaningful determinant of efficacy, and no loss of efficacy due to low plasma concentration was identified in clinical trials; therefore, TDM is unlikely to be provided during treatment with tofacitinib, according to a review published in 2021[10].

TDM can be implemented in two forms, "Proactive" TDM refers to routine monitoring of serum concentrations, whereas "Reactive" TDM is defined as a measurement taken following treatment failure. This allows doctors to then choose to adjust the dose or change to another drug. Cost-effectiveness is an important factor in the choice of proactive and reactive TDM. According to Assa *et al*[11], proactive monitoring and ADA dose intensification to serum concentrations > 5 μg/mL resulted in a higher rate of clinical remission than reactive monitoring in cases of "loss of response". Fernandes *et al*[12] and Papamichael *et al*[13] concluded that patients in the proactive TDM group had greater clinical outcomes than those in the control group. However, guidelines or consensus in different countries and regions differ on the application of TDM. Guidelines published in 2020 by the European Colitis & Crohn's Organization pointed out that there is insufficient evidence to recommend for or against TDM [14]. The 2017 guidelines of the American Gastroenterology Association only recommend reactive TDM [15]. McNeill *et al*[16] found that reactive TDM of IFX optimizes dosing and reduces expenditure by over 50%, without affecting clinical outcomes. Proactive IFX TDM may confer long-term clinical benefit but is only modestly cost-effective. A systematic review published in 2020 noted that compared with standard treatment without TDM, TDM-guided strategies were consistently reported to be cost saving or cost effective, with no emphasis on proactive or reactive TDM[17]. There are no high-quality studies comparing the cost-effectiveness of proactive and reactive TDM; however, TDM is cost-effective

compared to empirical treatment. The problems to be solved in the implementation of TDM include high price, delivery and transportation difficulties. A survey of over 242 participants in India suggested that significant barriers to TDM use were availability, cost and time lag for results. If these barriers were removed, almost all clinicians would use TDM at least reactively, and 25% would use it proactively[18].

It should be emphasized that the therapeutic goal is to achieve clinical and endoscopic remission and not to target TDM to specific drug concentration levels. Different guidelines recommend different trough concentrations, different departments have different measurement methods, and individuals have different systems, so even if the same threshold is reached, some people will respond and some will not; thus, we recommend dynamic detection of blood drug concentrations. The future medical trend is precision medicine and targeted medicine. We hope that in the future, there will be a tool as convenient and fast as a glucose meter that can measure blood drug concentrations, perform real-time monitoring, combine clinical symptoms and endoscopic manifestations, and then adjust drugs to achieve targeted treatment. We wish to draw readers' attention to the fact that TDM is a promising approach for clinicians to optimize treatment.

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FOOTNOTES

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REFERENCES

1   **Albader F**, Golovics PA, Gonczi L, Bessissow T, Afif W, Lakatos PL. Therapeutic drug monitoring in inflammatory bowel disease: The dawn of reactive monitoring. *World J Gastroenterol* 2021; **27**: 6231-6247 [PMID: 34712029 DOI: 10.3748/wjg.v27.i37.6231]

2   **Argollo M**, Kotze PG, Kakkadasam P, D'Haens G. Optimizing biologic therapy in IBD: how essential is therapeutic drug monitoring? *Nat Rev Gastroenterol Hepatol* 2020; **17**: 702-710 [PMID: 32879465 DOI: 10.1038/s41575-020-0352-2]

3   **van Hoeve K**, Seyed Tabib NS, Dreesen E, Tops S, Hoffman I, Gils A, Ferrante M, Vermeire S. Infliximab Concentrations during Induction Are Predictive for Endoscopic Remission in Pediatric Patients with Inflammatory Bowel Disease under Combination Therapy. *J Pediatr* 2022; **240**: 150-157.e4 [PMID: 34481805 DOI: 10.1016/j.jpeds.2021.08.079]

4   **Bar-Yoseph H**, Levhar N, Selinger L, Manor U, Yavzori M, Picard O, Fudim E, Kopylov U, Eliakim R, Ben-Horin S, Chowers Y, Ungar B. Early drug and anti-infliximab antibody levels for prediction of primary nonresponse to infliximab therapy. *Aliment Pharmacol Ther* 2018; **47**: 212-218 [PMID: 29124774 DOI: 10.1111/apt.14410]

5   **Kennedy NA**, Heap GA, Green HD, Hamilton B, Bewshea C, Walker GJ, Thomas A, Nice R, Perry MH, Bouri S, Chanchlani N, Heerasing NM, Hendy P, Lin S, Gaya DR, Cummings JRF, Selinger CP, Lees CW, Hart AL, Parkes M, Sebastian S, Mansfield JC, Irving PM, Lindsay J, Russell RK, McDonald TJ, McGovern D, Goodhand JR, Ahmad T; UK Inflammatory Bowel Disease Pharmacogenetics Study Group. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study. *Lancet Gastroenterol Hepatol* 2019; **4**: 341-353 [PMID: 30824404 DOI: 10.1016/S2468-1253(19)30012-3]

6   **Osterman MT**, Rosario M, Lasch K, Barocas M, Wilbur JD, Dirks NL, Gastonguay MR. Vedolizumab exposure levels and

clinical outcomes in ulcerative colitis: determining the potential for dose optimisation. *Aliment Pharmacol Ther* 2019; **49**: 408-418 [PMID: 30663076 DOI: 10.1111/apt.15113]

7   **Alsoud D**, Vermeire S, Verstockt B. Monitoring vedolizumab and ustekinumab drug levels in patients with inflammatory bowel disease: hype or hope? *Curr Opin Pharmacol* 2020; **55**: 17-30 [PMID: 33039940 DOI: 10.1016/j.coph.2020.09.002]

8   **Restellini S**, Afif W. Update on TDM (Therapeutic Drug Monitoring) with Ustekinumab, Vedolizumab and Tofacitinib in Inflammatory Bowel Disease. *J Clin Med* 2021; **10** [PMID: 33802816 DOI: 10.3390/jcm10061242]

9   **Afif W**, Sattin B, Dajnowiec D, Khanna R, Seow CH, Williamson M, Karra K, Wang Y, Gao LL, Bressler B. Ustekinumab Therapeutic Drug Monitoring-Impact on Clinical Practice: A Multicenter Cross-Sectional Observational Trial. *Dig Dis Sci* 2021 [PMID: 34401983 DOI: 10.1007/s10620-021-07173-1]

10   **Lee SD**, Shivashankar R, Quirk D, Zhang H, Telliez JB, Andrews J, Marren A, Mukherjee A, Loftus EV Jr. Therapeutic Drug Monitoring for Current and Investigational Inflammatory Bowel Disease Treatments. *J Clin Gastroenterol* 2021; **55**: 195-206 [PMID: 32740098 DOI: 10.1097/MCG.0000000000001396]

11   **Assa A**, Matar M, Turner D, Broide E, Weiss B, Ledder O, Guz-Mark A, Rinawi F, Cohen S, Topf-Olivestone C, Shaoul R, Yerushalmi B, Shamir R. Proactive Monitoring of Adalimumab Trough Concentration Associated With Increased Clinical Remission in Children With Crohn's Disease Compared With Reactive Monitoring. *Gastroenterology* 2019; **157**: 985-996.e2 [PMID: 31194979 DOI: 10.1053/j.gastro.2019.06.003]

12   **Fernandes SR**, Bernardo S, Simões C, Gonçalves AR, Valente A, Baldaia C, Moura Santos P, Correia LA, Tato Marinho R. Proactive Infliximab Drug Monitoring Is Superior to Conventional Management in Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2020; **26**: 263-270 [PMID: 31247074 DOI: 10.1093/ibd/izz131]

13   **Papamichael K**, Juncadella A, Wong D, Rakowsky S, Sattler LA, Campbell JP, Vaughn BP, Cheifetz AS. Proactive Therapeutic Drug Monitoring of Adalimumab Is Associated With Better Long-term Outcomes Compared With Standard of Care in Patients With Inflammatory Bowel Disease. *J Crohns Colitis* 2019; **13**: 976-981 [PMID: 30689771 DOI: 10.1093/ecco-jcc/jjz018]

14   **Torres J**, Bonovas S, Doherty G, Kucharzik T, Gisbert JP, Raine T, Adamina M, Armuzzi A, Bachmann O, Bager P, Biancone L, Bokemeyer B, Bossuyt P, Burisch J, Collins P, El-Hussuna A, Ellul P, Frei-Lanter C, Furfaro F, Gingert C, Gionchetti P, Gomollon F, González-Lorenzo M, Gordon H, Hlavaty T, Juillerat P, Katsanos K, Kopylov U, Krustins E, Lytras T, Maaser C, Magro F, Marshall JK, Myrelid P, Pellino G, Rosa I, Sabino J, Savarino E, Spinelli A, Stassen L, Uzzan M, Vavricka S, Verstockt B, Warusavitarne J, Zmora O, Fiorino G. ECCO Guidelines on Therapeutics in Crohn's Disease: Medical Treatment. *J Crohns Colitis* 2020; **14**: 4-22 [PMID: 31711158 DOI: 10.1093/ecco-jcc/jjz180]

15   **Mitrev N**, Vande Casteele N, Seow CH, Andrews JM, Connor SJ, Moore GT, Barclay M, Begun J, Bryant R, Chan W, Corte C, Ghaly S, Lemberg DA, Kariyawasam V, Lewindon P, Martin J, Mountifield R, Radford-Smith G, Slobodian P, Sparrow M, Toong C, van Langenberg D, Ward MG, Leong RW; IBD Sydney Organisation and the Australian Inflammatory Bowel Diseases Consensus Working Group. Review article: consensus statements on therapeutic drug monitoring of anti-tumour necrosis factor therapy in inflammatory bowel diseases. *Aliment Pharmacol Ther* 2017; **46**: 1037-1053 [PMID: 29027257 DOI: 10.1111/apt.14368]

16   **McNeill RP**, Barclay ML. Cost-effectiveness of therapeutic drug monitoring in inflammatory bowel disease. *Curr Opin Pharmacol* 2020; **55**: 41-46 [PMID: 33120169 DOI: 10.1016/j.coph.2020.09.006]

17   **Yao J**, Jiang X, You JHS. A Systematic Review on Cost-effectiveness Analyses of Therapeutic Drug Monitoring for Patients with Inflammatory Bowel Disease: From Immunosuppressive to Anti-TNF Therapy. *Inflamm Bowel Dis* 2021; **27**: 275-282 [PMID: 32311018 DOI: 10.1093/ibd/izaa073]

18   **Patel RN**, Nigam GB, Jatale RG, Desai D, Makharia G, Ahuja V, Limdi JK. An Indian national survey of therapeutic drug monitoring with anti-tumor necrosis (TNF) medications in inflammatory bowel disease. *Indian J Gastroenterol* 2020; **39**: 176-185 [PMID: 32483692 DOI: 10.1007/s12664-020-01047-6]

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