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Contents

Weekly Volume 30 Number 13 April 7, 2024

## EDITORIAL



## REVIEW



## MINIREVIEWS



## ORIGINAL ARTICLE

### Retrospective Cohort Study



### Retrospective Study

## Contents




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EDITORIAL

# Recent clinical trials and optical control as a potential strategy to develop microtubule-targeting drugs in colorectal cancer management

Katsuhiro Kita, Allen Burdowski

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**Katsuhiro Kita, Allen Burdowski,** Department of Biology, St. Francis College, Brooklyn, NY 11201, United States

**Corresponding author:** Katsuhiro Kita, PhD, Assistant Professor, Department of Biology, St. Francis College, 179 Livingston Street, Brooklyn, NY 11201, United States. kkita@sfc.edu

## Abstract

Colorectal cancer (CRC) has remained the second and the third leading cause of cancer-related death worldwide and in the United States, respectively. Although significant improvement in overall survival has been achieved, death in adult populations under the age of 55 appears to have increased in the past decades. Although new classes of therapeutic strategies such as immunotherapy have emerged, their application is very limited in CRC so far. Microtubule (MT) inhibitors such as taxanes, are not generally successful in CRC. There may be some way to make MT inhibitors work effectively in CRC. One potential advantage that we can take to treat CRC may be the combination of optical techniques coupled to an endoscope or other fiber optics-based devices. A combination of optical devices and photo-activatable drugs may allow us to locally target advanced CRC cells with highly potent MT-targeting drugs. In this Editorial review, we would like to discuss the potential of optogenetic approaches in CRC management.

**Key Words:** Colorectal cancer; Chemotherapy; Microtubule; Combretastatin; Photopharmacology

**Core Tip:** This review article proposes a potentially new approach to utilize photo-switchable microtubule (MT)-targeting drugs in colorectal cancer (CRC). First, we will start the introduction of CRC and current therapy as well as some updates in 2023. Then, we list a popular MT-targeting drug family, taxanes in CRC. As many readers may be aware, taxanes are not really effective in CRC for some reason. Here, we would like to shed light on optically controllable MT-targeting drugs as potential new drug candidates in CRC management.

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## INTRODUCTION

Currently, colorectal cancer (CRC) is the third leading cause of cancer-associated death in the United States and the second worldwide. Based on the most updated CA report, 153020 estimated new cases of CRC in the United States this year (2023)[1]. The overall CRC mortality decreased from 29.2 per 100000 (1970) to 12.6 per 100000 (2020). In addition, mortality decline has surpassed incidence, because improved treatments extended patient survival. Although the age-standardized incidence of CRC has decreased by nearly 50% in 2019, compared to the incidence in 1985, a rapid increase of advanced CRC in the range of 20-50 years old is alarming, because this is the socially very active age group that also contributes to growing the next generation by having families. As widely introduced to the public earlier this year by news media[2,3], this trend gets the attention of the general public. Thus, although the diagnosis and treatments have been significantly developed during the past two decades, it is still very important to us to further develop more effective treatments for CRC, with a primary focus on advanced CRC. As the early detection of CRC has improved the overall survival (OS) rate up to 91%, improvement of the survival rate in the late-stage, such as stage IV CRC is the one that more efforts may be needed.

As summarized in a recent systematic review[4], the data from a total of 150 phase III clinical trials between 1986-2016 indicates that only 35 of 132 trials (26.5%) showed improved OS of patients more than two months. This summary clearly indicates that there are still many conditions to be addressed to significantly improve clinical outcomes. A nucleotide analog (fluorouracil) and a platinum drug (oxaliplatin) have been the mainstay in the first-line chemotherapy of CRC[5]. In addition, others such as a topoisomerase inhibitor (irinotecan)[6] and a pro-drug of fluorouracil (capecitabine) are also used[7,8]. However, microtubule (MT)-targeting drugs, such as taxanes, are not included in the current standard regimens for CRC.

In this review article, we would like to begin with a summary of the current standard treatment options and very recent examples [combination of vascular endothelial growth factor (VEGF) receptor signaling inhibition]. Then, we move on to discuss possible reasons why the MT cytoskeleton may not be a successful therapeutic target in CRC. Although cancer immune therapy is certainly one of the promising approaches to expanding the applications to CRC, cancer immunotherapy has also not been greatly successful in CRC to date. We would like to discuss a new class of MT inhibitors and their potential combination with light–"photopharmacology" toward the end.

## CURRENT FIRST-LINE THERAPY OF CRC IN THE UNITED STATES

Because the high mortality of CRC is caused by late-stage CRC, a particular focus on the management of metastatic CRC (mCRC) would be critical to improving the OS of CRC patients. Based on the Centers for Disease Control and Prevention's recent data, the current 5-year relative OS of mCRC is 15% (CDC https://seer.cancer.gov/statfacts/html/colorect.html). In addition to the traditional fluorouracil-based therapy (5-FU), a recent recommendation by the American Society of Clinical Oncology's expert panel summarized more targeted therapy options based on molecular characteristics [9]. This guideline suggests doublet or triplet chemotherapy, depending on the subtypes of mCRC (Ras wild-type, BRaf V600E mutant) and certain microsatellite stable or proficient mismatch repair types. Briefly speaking, more personalized options are recommended in CRC management.

## NEW COMBINATION THERAPIES IN 2023

There are a few newly reported combination chemotherapy regimens to treat refractory CRC in 2023. One of the reports was SUNLIGHT trial[10] utilizing the combination of bevacizumab (Avastin), trifluridine (FTD) and tipiracil (TPI; Lonsurf)[11]. The previously reported FTD-TPI combination, the third-line treatment option, already showed significant improvement in patient OS[12]. The addition of a humanized anti-VEGF-A antibody, bevacizumab[13], resulted in 144% increases in median OS (from 7.5 to 10.8 months) and a 230% increase in progression-free survival (2.4 to 5.6 months)[11]. These numbers are very significant improvements in advanced CRC management. FDA approved this combination

therapy in August, 2023[14]. We should note that bevacizumab can also inhibit angiogenesis; contraindication was reported among patients receiving bevacizumab, and lung and colon cancer patients have higher chances of contraindications based on the search of patients older than 65 years old[15].

The other notable targeted therapy reported in 2023 was FRESCO-2 trial using fruquintinib[16]. In this study, fruquintinib, a potent and orally administrable VEGF receptor tyrosine kinase inhibitor[17], was shown to improve median OS to 7.4 months compared to the placebo group (4.8 months). Although effective, we should note that grade 3 or worse adverse events were observed in 63% of the group receiving fruquintinib.

In summary, it is very clear that the combination of traditional DNA binding drugs and targeting of the VEGF signaling has shown superior OS in refractory/mCRC. However, we should carefully monitor the adverse effects associated with the inhibition of VEGF signaling.

## LONG WAY TO DEVELOPING EFFECTIVE IMMUNOTHERAPY FOR CRC

Cancer immune therapy is one of the areas that is recently expanding its application to a variety of tumors. In CRC, an immune checkpoint inhibitor, pembrolizumab (Keytruda), is the only FDA-approved cancer immunotherapy drug for CRC so far (approved by FDA in 2017[18]). Theoretically, programmed cell death-1 (PD-1) blockage should be effective in helping CD8+ cytotoxic T cells target all tumor cells. Currently, high microsatellite instability/deficient mismatch repair (MSI-H/dMMR) locally advanced CRC patients may receive this treatment. In fact, a 2019 study showed increased programmed cell death-ligand 1 (PD-L1) expression in the tumor microenvironment of MSH-H/dMMR patients, suggesting that immune checkpoint inhibitors may be a great therapeutic target[19].

Since the rapid development of CRISPR-Cas9 technology, the use of chimeric antigen receptor-T (CAR-T) cells is the other strategy to target specific tumor-associated antigens[20]. Although a total of 25 CAR-T cell clinical trials for CRC are ongoing as of 2022[21], the application of CAR-T cell therapy to CRC faces a challenge because of the limited infiltration of CAR-T cells to the local tumor tissues, as discussed in detail[20]. This lack of infiltration is a major challenge in the application of CAR-T therapy to solid tumors in general. Unlike the successful approval of CAR-T therapy in hematopoietic malignancies[22], it will take a while until we find a way to successfully apply CAR-T therapy in CRC.

## WHY TAXANES MAY NOT WORK WELL IN CRC

The MT-targeting drug, represented by taxanes, are well-established chemotherapeutic drug as represented by breast cancer treatment[23]. Besides breast cancer, paclitaxel and its derivatives are also a choice in ovarian, prostate, non-small cell lung, and gastric cancer[24]. However, MT-targeting drugs are not included in CRC chemotherapeutic regimens. One well-acknowledged fact is that CRC shows resistance to a wide spectrum of chemotherapeutic drugs, probably due to relatively high levels of P-glycoprotein. Compared to the spleen, stomach, ovary, skin, and lymphocytes, it was reported that the P-glycoprotein mRNA expression level in the colon is 6-30 times higher[25]. Thus, it makes sense that platinum-based DNA-targeting drugs are used as the first-line chemotherapeutic drugs in late-stage colon cancer management because platinum-based chemotherapeutic drugs covalently bind to DNA.

Table 1 summarizes the currently recruiting clinical trials including paclitaxel that target CRC. There are 13 trials (note that two of them study peritoneal carcinomatosis and anal cancer) and all others investigate combinational therapies. A small cohort of a phase II clinical trial conducted by MD Anderson Cancer Center's group tested the efficacy of albumin-conjugated paclitaxel (nab-paclitaxel)[26] on CRC as well as small bowl adenocarcinoma[27]. Although the trial demonstrated a promising result[25]. What we should note is that this clinical trial focused on a subset of CRC–CpG island methylator phenotype–and small bowl adenocarcinoma.

The doubling time of CRC cells is expected to be very long. The median doubling time of tumor volume was 130 d[28], thus, it would be fair to postulate that the doubling time of colorectal tumor cells may not be fast *in vivo*, unlike culture cell lines. This could be the other reason why MT-targeting drugs such as paclitaxel do not work well to treat CRC.

Docetaxel (Taxotere) is a semisynthetic analog of paclitaxel that was reported in 1991[29,30]. Because of its more potent activity, docetaxel has been quickly tried in many solid tumors. Nearly two decades ago, a phase II trial of docetaxel in mCRC was concluded[31]. Based on this trial, docetaxel showed little effect on mCRC treatment, unlike ovarian, breast, and non-small cell lung cancer. We do not know why the mouse CRC model system showed a very promising result[30]. Thus, basically, docetaxel is not recognized as an effective chemotherapic agent for CRC management[32]. Although docetaxel monotherapy may not be an option in CRC, it should be noted that there are a few potentially interesting experimental studies; RasSF10 suppresses CRC growth by activating p53 signaling to sensitize CRC cells to docetaxel[33], and this year, there is another study reporting the co-delivery of Akt inhibitor and docetaxel to CRC utilizing the CD44-targeted nanoparticles[34]. Thus, docetaxel may give more new combination therapy options than paclitaxel in the future. Table 2 summarizes the currently recruiting clinical trials including docetaxel in CRC. There is no phase III trial including docetaxel, and all recruiting trials add docetaxel as a part of the design.

Cabazitaxel is a more recently added, semi-synthetic paclitaxel analog that was approved by the FDA for the treatment of hormone-refractory metastatic prostate cancer[35]. Only one report has shown the suppression of CRC cell growth by activating p53 (using HCT116, LOVO, HCT8, and DLD1 cell lines as well as a xenograft model using HCT116)[36]. Thus, there is a potential to investigate the effectiveness of cabazitaxel in clinical settings, however, so far there are no cabazitaxel clinical trials for CRC.

**Table 1 Recruiting colorectal cancer clinical trials that include paclitaxel**

| Clinical trial number | Treatment | Stage | Country |
|---|---|---|---|
| NCT05185947 | Paclitaxel + nilotinib | Phase II | United States |
| NCT04731467 | Nivolumab, Nab-paclitaxel, gemcitabine | Phase I/II | United States, Spain |
| NCT03129139 | Minnelide™ + protein-bound paclitaxel | Phase I | United States |
| NCT05453825 | Navicixizumab + paclitaxel or irinotecan | Phase II | United States |
| NCT05107674 | NX-1607 (+ paclitaxel) | Phase Ia/b | United States, United Kingdom |
| NCT03678883 | 9-ING-41, gemcitabine, doxorubicin, carboplatin, Nab-paclitaxel, paclitaxel, irinotecan | Phase II | United States, Belgium, Canada, France, Netherlands, Portugal, Spain |
| NCT05395910 | Paclitaxel | Phase I | Singapore (note: Peritoneal carcinomatosis) |
| NCT04666688 | LYT-200, tislelizumab, gemcitabine + Nab-paclitaxel | Phase I/II | United States |
| NCT04444921 | Carboplatin, nivolumab, paclitaxel | Phase III | United States (note: Anal cancer) |
| NCT04083599 | GEN1042, pembrolizumab, cisplatin, carboplatin, 5-FU, gemcitabine, Nab-paclitaxel, pemetrexed, paclitaxel | Phase I/II | United States, Denmark, France, Georgia, Germany, Israel, Italy, Republic of Korea, Republic of Moldova, Spain, Taiwan, United Kingdom |
| NCT03872947 | TRK-950, irinotecan, leucovorin, 5-FU, gemcitabine, cisplatin, carboplatin, ramucirumab, paclitaxel, nivlumab, pembrolizumab, imiquimod cream, bevacizumab, topotecan, PCD | Phase I | United States, France |
| NCT04644068 | ADZ5305, paclitaxel, carboplatin, T-Dxd, Dato-DXd, camizestrant | Phase I/II | United States, Australia, Canada, China, Czechia, Hungary, Italy, Japan, Republic of Korea, Poland, Russian Federation, Spain, United Kingdom |
| NCT06047379 | NEO212, ipilimumab, pembrolizumab, nivolumab, regorafenib, carboplatin, paclitaxel, FOLFIRI, bevacizumab | Phase I/II | United States |

**Table 2 Recruiting docetaxel study in colorectal cancer**

| Clinical trial number | Treatment | Stage | Country |
|---|---|---|---|
| NCT04553692 | IGM-844 + FOLFIRI (+ bevacizumab) (docetaxel included as a part) | Phase I | United States |
| NCT04256707 | Selinexor, docetaxel, pembrolizumab, FOLFIRI | Phase I/II | Israel |
| NCT02817633 | TSR-022, nivolumab, TSR-042, 033, docetaxel, pemetrexed, cisplatin, carboplatin | Phase I | United States |
| NCT05714553 | Fosifloxuridine nafalbenamide, leucovorin, pembrolizumab, docetaxel | Phase I/II | United Kingdom |
| NCT04895709 | BMS-986340, 936558-01, docetaxel | Phase I/II | United States |
| NCT04894370 | (Sample collection) | Phase II | France |

In summary, taxanes have not been effective in treating CRC and thus have not been used as a choice in CRC management, although some clinical trials are going on, to combine either paclitaxel or docetaxel with other agents.

It is very interesting to note that sets of chemotherapy-induced gene expression signatures and the OS were correlated in breast cancer but not in CRC cells[37]. Cytokine responses to chemotherapeutic agents may be one of the potentially useful parameters to predict CRC to chemotherapy.

## COMBRETASTATIN: A POTENT MT INHIBITOR

Based on the past research including clinical trials, MT is not a major target for the management of CRC. There is also a group of drugs that destabilize MTs, as represented by nocodazole. Nocodazole is mainly used to study MT functions and dynamics in cell biology–besides nocodazole, combretastatin might be a very interesting drug to study. Based on the information on medicinal and poisonous plants in Africa, the isolation of the first combretastatin was reported in 1982 [38], followed by several studies reporting structural analogs[39-41]. Originally isolated from an African tree, *Combretum*

*caffrum*, combretastatin is known as a very potent MT polymerization inhibitor[42]. Regardless of its discovery in the late 1980s, few applications of combretastatin have been studied until the 21st Century, maybe partly because of its very potent cytotoxicity. As seen in Table 3, there are only 19 clinical trials investigating combretastatin in all cancers (all are completed or terminated trials). It appears that the risk of adverse events is high. The high toxicity of combretastatin may probably be a part of the reasons that have hindered the application of combretastatin as a potential chemotherapeutic drug so far. Unfortunately, there is no combination trial of combretastatin in CRC; one clinical trial in Table 3[43] in anaplastic thyroid cancer shows that approximately 2-fold higher serious adverse events in the arm including CA-4 (+carboplatin and paclitaxel; 41.18%) compared to the control arm (carboplatin and paclitaxel only; 20.83%). The survival of combretastatin-including arm is 5.2 months (range: 3.1-9.0) and carboplatin and paclitaxel-only is 4.0 months (2.8-6.2), respectively. Thus, although the inclusion of combretastatin may slightly increase the survival period of patients, there is no statistically significant difference between both groups ($P$ = 0.223). This, apparently no statistically significant difference, might be partly because of the relatively small scale of the study.

So, what would be the other option to develop CA-4-based therapy? Traditional development of natural compounds-based drugs will start by accomplishing the purification of the original, natural products[44]. From that point, total chemical synthesis of the original molecules will be tried, and then a variety of analogs will be synthesized to evaluate biological activities. More recently, biosynthetic bioengineering has also been considered as an alternative strategy[45]. CA-4 is not an exception. As reviewed by Hamze *et al*[46], there have been an astonishing 131 CA-4 analogs synthesized and tested from 2009 to 2019. Importantly, 114 out of 131 were tested with CRC cell lines (mostly HCT116). As detailed in this review, the natural CA-4 (*Z* (*cis*)-isomer) can be converted to the *E* (*trans*)-isomer by isomerization. The *E*-isomer is much less effective ($GI_{50}$ is 80 times higher than *Z*-form). To prevent isomerization, the synthesis of a stable, non-natural isomer, *iso*CA-4 was reported by the authors' group in 2009[47,48]. The replacement of *cis*-/*trans*-bond between two trimethylphenyl rings with 1,1-diarylethylene structure made it possible to avoid isomerization meanwhile keeping the almost identical $GI_{50}$. Thus, newly developed panels of *iso*CA-4 analogs may be very promising as a monotherapy or combination therapy candidate.

More recently, CA-4 was shown to downregulate VEGF signaling *via* two different mechanisms: (1) Suppression of VEGF secretion in HUVEC as well as MCF-7 cell lines; and (2) VEGFR-2 expression and activation. Thus, CA-4 molecule itself already has two distinct activities: (1) MT destabilization; and (2) Anti-angiogenic activity[49]. This is essentially the same mechanism of action as the combination of paclitaxel (except paclitaxel polymerizes MT instead) and navicixizumab tried in ovarian cancer[50,51]. Therefore, the development of CA-4-based treatments may be still worth considering. In fact, a study using a mouse xenograft model showed that both the tumor growth curve, as well as the xenograft weight, were significantly reduced by the combination of CA-4[52].

## OPTICALLY CONTROLLABLE COMBRETASTATIN

There have been some trials developing the area of "photo-pharmacology" utilizing photo-switchable drugs or light-induced activation of pro-drugs[53]. Because of its chemical structure, combretastatin molecules can exist in two different forms as described in the previous section; *i.e.*, *Z*- and *E*-forms. This means that the double-bond connecting two trimethylphenyl rings can result in interexchangeable *cis*- and *trans*-isomers. Because the *trans*-isomer is 80 times less effective in suppressing CRC cell growth, this isomerization has been reported to occur during storage and metabolism. Besides high toxicity, this is a potential pitfall–yet if we can control the conformation locally, CA-4 or its analogs may be very effective anti-cancer chemotherapeutic drug candidates.

Pro-drug would be one of the strategies to locally activate a drug. The first prodrug of CA-4 was reported in 2013[54]. In this study, the authors successfully convert the prodrug dithiaporphyrin-aminoacylate-CA-4 (CMP-L-CA-4) into CA-4 using 690 nm diode laser irradiation. The study used both breast cancer cell lines as well as an *in vivo* mouse model system. It is also notable that over 80% of this photo-cleavable CMP-L-CA-4 can release CA-4 in 10 min. $IC_{50}$ was increased 6-fold after the irradiation[54]. The same group also developed different versions of prodrugs (Pc-(L-CA-4)$_2$ and Pc-(NCL-CA-4)$_2$). In those cases, approximately 26-28-fold increase in cytotoxicity was observed upon the release of CA-4 [55]. Although both are great photoactivatable CA-4, inactive prodrugs are only 6-28 times less toxic compared to CA-4. Thus, it is desired to keep inactive forms of CA-4 less toxic. Then, in 2015, photoswitchable photostatins (PST) were reported by the Trauner and Thorn-Seshould labs[56]. All PSTs replaced the C=C double bond connecting two trimethoxybenzene rings with the N=N double bond to give the azobenzene PSTs. In this study, the authors showed the comparison of eight different PSTs. One of them, the azologue of CA-4 phosphate, showed the best activation (101-fold activation upon photo-isomerization). Although all PSTs give μM range of $EC_{50}$ as *trans*-forms (inactive), the majority of *cis*-form (active) PSTs showed sub-μM level $EC_{50}$. This is a very promising result, and supported by a few cell line-based experiments including poly(ADP-ribose) polymerase cleavage (a hallmark of apoptosis). Although spontaneous *cis* to *trans* isomerization occurs over minutes, a 75 ms pulse every 15 s can convert *trans* form to *cis* form quickly. More importantly, the authors confirmed the stability of PSTs–over 5000 times switches over two days. Thus, overall, PSTs are potentially promising, photoactivatable CA-4.

As discussed by Mulatihan *et al*[57], azobenzene reductase and NAD(P)H could cleave the N=N double bond in PSTs. Thus, it may be important to measure the elevation of azobenzene reductase level in experimental settings. Hypoxia is common in tumor microenvironment, and thus we may not underestimate azobenzene reductase's effect.

**Table 3 The summary of clinical trials investigating CA-4 in cancer treatment**

| Clinical trial number | Conditions | Trial stage | Enrollment | Study period |
|---|---|---|---|---|
| NCT01570790 | Age-related macular degeneration | Phase I/II | 8 | 2003-2005 |
| NCT01423149 | Myopia | Phase II | 23 | 2005-2007 |
| NCT00060242 | Head and neck cancer | Phase II | 26 | 2003-2008 |
| NCT00113438 | Cancer (non-specified) | Phase II | 23 | 2005-2007 |
| NCT00960557 | Neoplasm metastasis | Phase I | 16 | 2009-2010 |
| NCT00077103 | Head and neck cancer | Phase I/II | 4 | 2003-2007 (terminated) |
| NCT00003768 | Adult solid tumor (unspecified) | Phase I | 25 | 1998-2001 |
| NCT00507429 | Anaplastic thyroid cancer | Phase II/III | 80 | 2007-2011 (terminated) |
| NCT01023295 | Polypoidal choroidal vasculopathy | Phase II | 20 | 2009-2010 |
| NCT00395434 | Tumors (unspecified) | Phase I | 20 | 2006-2007 |
| NCT02576301 | Acute myelogenous leukemia/myelodysplastic syndromes | Phase I/II | 105 | 2015-2020 (status unknown) |
| NCT00003698 | Adult solid tumor (unspecified) | Phase I | 35 | 1998-2003 |
| NCT01085656 | Acute myelogenous leukemia/myelodysplastic syndromes | Phase I | 18 | 2011-2016 (terminated) |
| NCT02279602 | Neuroendocrine tumors | Phase II | 7 | 2014-2016 |
| NCT00699517 | Sarcoma | Phase III | 355 | 2008-2013 |
| NCT02132468 | Neuroendocrine tumors | Phase II | 18 | 2014-2016 |
| NCT01701349 | Anaplastic thyroid cancer | Phase III | 0 | 2015-2017 (withdrawn) |
| NCT00653939 | Non-small cell lung cancer | Phase II | 63 | 2008-2011 |
| NCT02641639 | Platinum-resistant ovarian cancer | Phase II/III | 91 | 2016-2017 (terminated) |
| NCT01240590 | Anaplastic thyroid cancer | Phase I/II | 27 | 2011-2016 |

## DEVELOPMENT OF OTHER PHOTO-SWITCHABLE MT-TARGETING DRUGS

Because of its chemical structure (Figure 1), combretastatin is probably the easiest MT-targeting drug to control with light. Briefly speaking, there has to be one double bond ($R_1$-C=C-$R_2$ or $R_1$-N=N-$R_2$) to allow the conformational switching (*i.e.*, *cis vs trans*). It is very challenging to develop photo-switchable derivatives of other MT-targeting drugs, such as taxanes, because taxanes do not contain a C=C double bond suitable to induce photo-isomerization. Nevertheless, a few photo-switchable taxanes were developed very recently. The first report was published in 2020[58]–it used paclitaxel as the base and added azobenzene to one of the side chains because taxanes have no double bonds in their core structures that allow photo-isomerization. In this first report, careful selection of the modified side chain (Figure 2, top arrow) was made based on the distance of side chains from paclitaxel's target, β-tubulin. Because a fluorescent dye-conjugated taxane was made by coupling a fluorophore at the 3'-amino group[59], and such fluorescently label taxanes still retained the ability to bind to MTs. Among 10 derivatives that the authors synthesized, the substitution of the benzamide side chain of paclitaxel with $OCH_3$-azobenzene (in *meta* configuration) gave the most effective compound, AzTax3MP (Figure 2, top). AzTax3MP changes the $EC_{50}$ from 1.4 to 0.24 μM upon shining with 360 nm LED light[58]. This research is certainly a great pharmacological advance, as paclitaxel has been used in several types of solid tumor management for a long period of time[24]. One potential disadvantage is the dynamic range of the toxicity before and after photo-switching. Photo-switching only increases the $IC_{50}$ by less than 6-fold. The following study[60] initially attempted the development of a photo-switchable docetaxel analog (SBTax) by conjugating SBTub to docetaxel. To create SBTax, the addition of the styrylbenzothiazole needed to be conjugated to the amino group of the side chain of docetaxel (Figure 2, bottom arrow). However, the low solubility and bioactivity of SBTax precluded it from an ideal photo-switchable MT drug in this case.

In the same study[60], the authors then explored the synthesis of photo-switchable epothilone analogs. The authors also pointed out the potential advantages of epothilones because of their high solubility in water and the ability to cross the blood-brain barrier. Although epothilones have one C=C double bond at the side chain, this double bond may not be suitable for photo-isomerization because it is adjacent to the core ring structure. Therefore, the installation of an extending photo-switchable group was tried. Among several modifications, the author mentioned that the compound STEpo2 (styrylthiazole group coupled to the core structure of epothilone D; Figure 3) showed better synthetic access and the highest cytotoxicity upon photo-switch ($IC_{50}$ = 0.1 μM). It appears that STEpo2 and a few derivatives can show up to 4-fold potency change.

In summary, very recently, photo-switchable compounds were derived from clinically-proven MT-targeting drugs. However, right now, the dynamic range of potency shift is not comparable to what is seen in combretastatin analogs (combretastatin and its photo-switchable derivatives showed a 60-100+-fold potency shift). In addition, taxanes were not

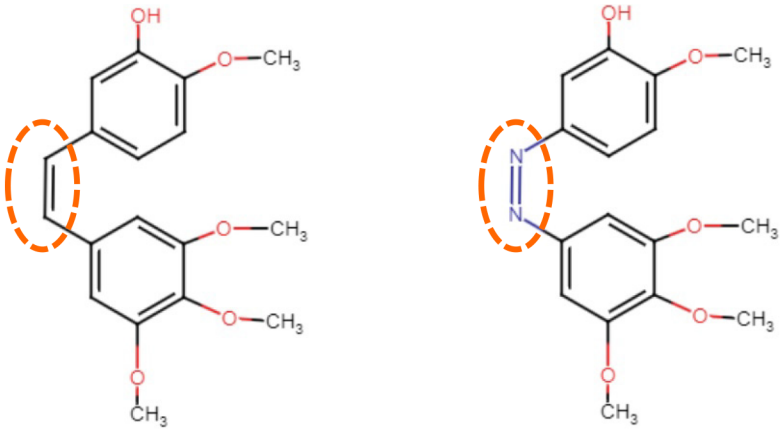

**Figure 1 The chemical structures of combretastatin CA-4 (left) and photostatin photoswitchable photostatins-1[56].** Both compounds have either the C=C or N=N double-bond that can induce photo-isomerization by light (circle).

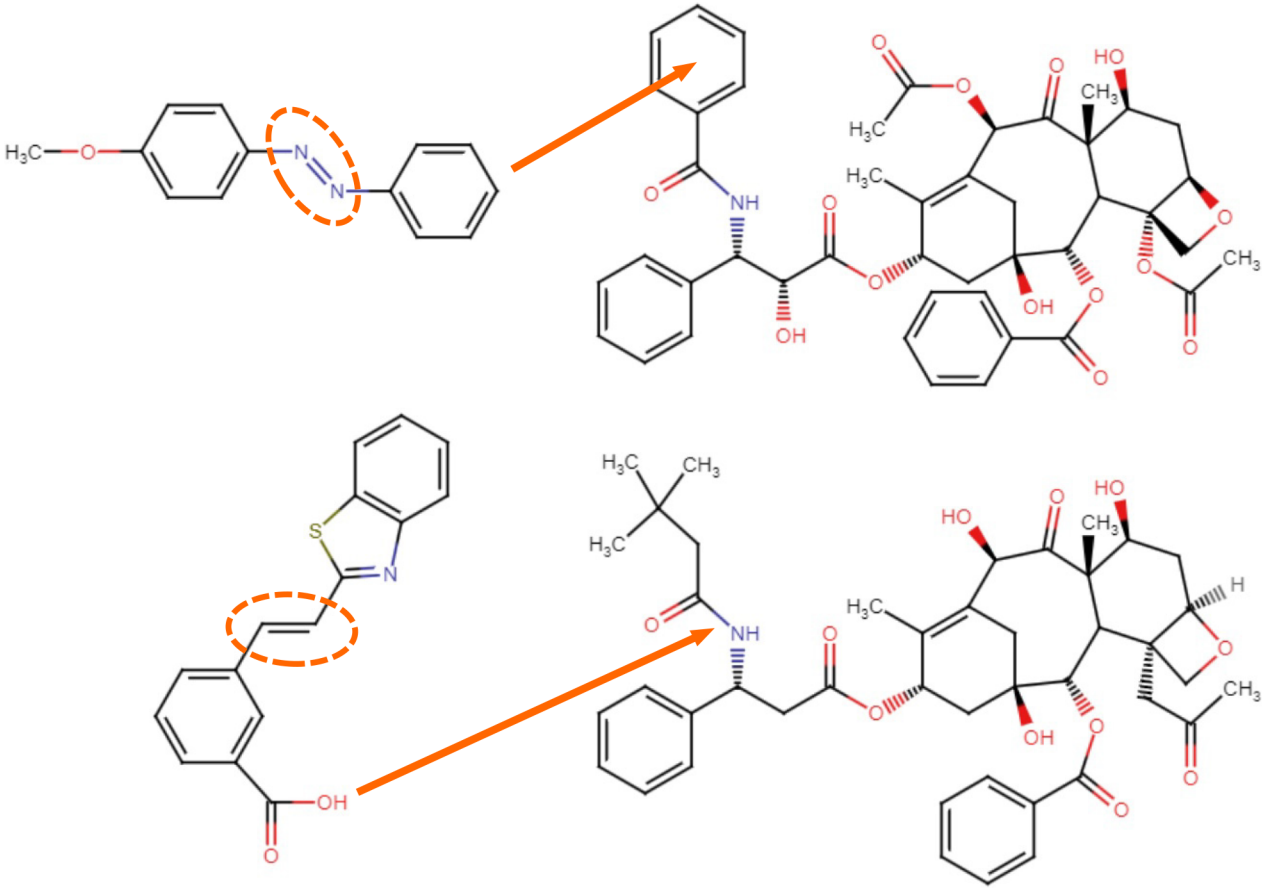

**Figure 2 The chemical structures of paclitaxel (top right) with an azobenzene derivative (top left) and docetaxel with a styrylbenzothiazole derivative (bottom).** The photo-switchable C=C or N=N double-bond is highlighted (dotted circle). Each group is conjugated to the left arm of taxanes (arrows), resulting AzTax3MP[58] and SBTax[60], respectively.

successful in CRC management in past clinical trials. Therefore, we think that combretastatin analogs might hold better promise to develop clinically applicable, photo-controllable drug candidates.

## DEVELOPMENT OF ENDOSCOPY CAPABLE OF HANDLING TWO-PHOTON ILLUMINATION

To convert photo-switchable combretastatin to the active form, a relatively short wavelength (390 nm) is required. Although it is not a UV range, a longer wavelength is preferred to avoid DNA damage. Two-photon excitation would be a great solution to avoid damages generated by short wavelengths, and more importantly, longer, near-infrared red light

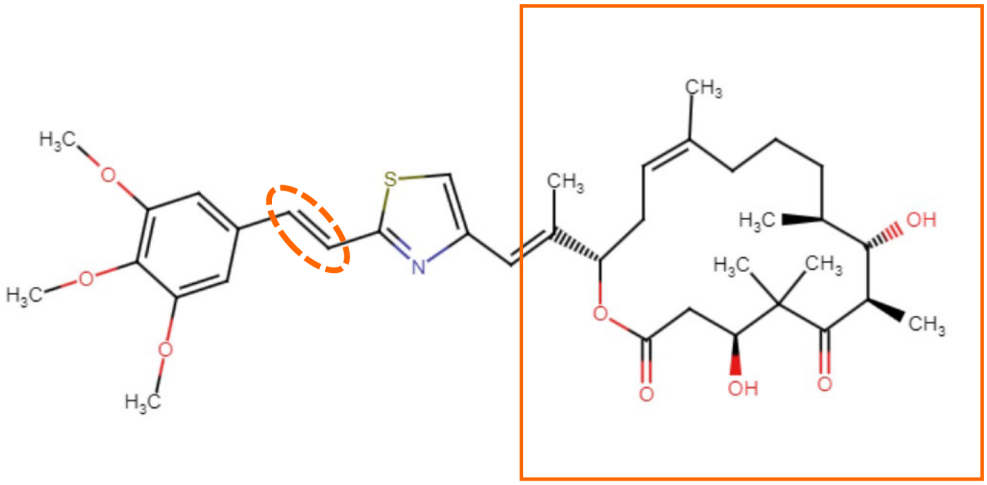


**Figure 3 The chemical structures of STEpo2[60], a photo-switchable derivative of epothilone D.** The photo-switchable C=C double-bond (dotted circle) in the attached styrylthiazole group. The rectangle indicates the core structure of epothilone D.

can penetrate deep inside tissue. The first two-photon fluorescence microscopy was reported by Denk *et al*[61]. However, two-photon excitation utilizes a giant femto-second pulse laser, and thus initially it was very difficult to apply two-photon excitation to *in vivo* applications. The development of optical fiber technologies enables us to guide the two-photon excitation beam into tissues[62-65]. This development allowed the application of two-photon excitation for colon cells[66], and the first clinical two-photon endoscope was reported in the same year[67]. There is no report trying the photo-activation of CA-4 analogs *in vivo* so far. We look forward to seeing CA-4-based photopharmacology.

To our knowledge, the first report of a photopharmacological approach reporting the photo-activation of a prodrug appeared in 2013[68]. The study showed local activation of doxorubicin prodrug using a mouse model system and fiber optics. The mixture of A549 human non-small cell lung cancer cells and matrigel were injected into flanks of nude mice, thus, the condition may be a little artificial. This doxorubicin prodrug is cleaved and releases the active, doxorubicin. One very important message from this preliminary study is that: (1) Doxorubicin was not detected in serum before the local photoactivation; and (2) After photoactivation, doxorubicin stayed a little (30 min after light exposure) in the local tissue and was not immediately detected in the collected serum. Thus, the concept of "photopharmacology" was shown *in vivo* for the first time in 2013.

Although it is not related to combretastatins, endoscope-assisted phototherapy was carried out using a photoswitchable proteasome inhibitor and HCT-116 human CRC cell line recently[69]. Thus, photopharmacology was achieved with a CRC cell line model system.

## CONCLUSION

Because of the increasing incidence of CRC in younger age groups, it is increasingly important to further develop better treatment options for CRC, especially mCRC. Current first-line therapy regimens have been built around platinum and nucleotide analogs, yet the combination of other signaling, such as VEGF signaling inhibitors, showed promising results in recent clinical trials. Because of its role in fundamental biological processes, MT should be an important target in clinical oncology, as we have seen in other solid tumor management. However, MT inhibitors alone have not been successful in CRC management. Currently, there are clinical trials investigating the combinations of taxanes and other agents in CRC. Meanwhile, we should look forward to the other promising MT inhibitors. Here, we reviewed some promising recent clinical trials, mainly promising combination therapies. Our main interest is how we can develop MT-targeting drugs more effective for cancer types that did not show good clinical responses in the past. Then we introduced one potential solution that may allow effective, local activation of drugs using light–photopharmacology. Light-induced isomerization has great potential to achieve local activation of very potent drugs, which are usually too harmful, such as combretastatin. The unique chemical structure of compbretastatin CA-4 and the developed prodrugs and PSTs also showed a very effective potency shift[56] that is promising to further develop them for pre-clinical applications. Although more recent studies attempted to synthesize photo-switchable taxanes and epothilone derivatives[58,60], their narrower potency shift may make them less ideal right now for pre-clinical applications. With the recent advance of fiber optics and a proof-of-concept study, it may be time to design a new MT drug with the power of light.

## FOOTNOTES

**Author contributions:** Kita K and Burdowski A conceived the idea and wrote the manuscript.

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**Country/Territory of origin:** United States

**ORCID number:** Katsuhiro Kita 0000-0001-7392-1722.

**Corresponding Author's Membership in Professional Societies:** American Society for Cell Biology, No. 43778.

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EDITORIAL

# Cellular strategies to induce immune tolerance after liver transplantation: Clinical perspectives

Ai-Wei Zhou, Jing Jin, Yuan Liu

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**Ai-Wei Zhou, Yuan Liu,** Department of Liver Surgery, Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

**Jing Jin,** Department of Nursing, Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

**Yuan Liu,** Department of Liver Transplantation, Shanghai Immune Therapy Institute, Shanghai 200127, China

**Corresponding author:** Yuan Liu, MD, Assistant Professor, Department of Liver Surgery, Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, No. 160 Pujian Road, Shanghai 200127, China. liuyuanbird@163.com

## Abstract

Liver transplantation (LT) has become the most efficient treatment for pediatric and adult end-stage liver disease and the survival time after transplantation is becoming longer due to the development of surgical techniques and perioperative management. However, long-term side-effects of immunosuppressants, like infection, metabolic disorders and malignant tumor are gaining more attention. Immune tolerance is the status in which LT recipients no longer need to take any immunosuppressants, but the liver function and intrahepatic histology maintain normal. The approaches to achieve immune tolerance after transplantation include spontaneous, operational and induced tolerance. The first two means require no specific intervention but withdrawing immunosuppressant gradually during follow-up. No clinical factors or biomarkers so far could accurately predict who are suitable for immunosuppressant withdraw after transplantation. With the understanding to the underlying mechanisms of immune tolerance, many strategies have been developed to induce tolerance in LT recipients. Cellular strategy is one of the most promising methods for immune tolerance induction, including chimerism induced by hematopoietic stem cells and adoptive transfer of regulatory immune cells. The safety and efficacy of various cell products have been evaluated by prospective preclinical and clinical trials, while obstacles still exist before translating into clinical practice. Here, we will summarize the latest perspectives and concerns on the clinical application of cellular strategies in LT recipients.

**Key Words:** Cellular therapy; Induced tolerance; Liver transplantation; Regulatory T cells; Regulatory dendritic cells

**Core Tip:** Immune tolerance after liver transplantation could significantly reduce the long-term side-effects of immunosuppressants. Compared with operational and spontaneous tolerance, induced tolerance by cellular therapy could reduce immunosuppressant dosage at early stage after transplantation. Regulatory immune cells could suppress the inflammatory response, which are widely explored in preclinical and clinical trials. So far, regulatory CD4+ T cells, mesenchymal stromal cells and regulatory dendritic cells are mostly studied. However, even the safety and tolerability of cellular therapy in transplantation recipients have been validated, the overall efficacy of tolerance induction is unsatisfactory. Detailed exploration is required in the future.

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## INTRODUCTION

With development of surgical techniques and perioperative management, liver transplantation (LT) has become the most efficient treatment for end-stage liver diseases, with 75%-90% recipients owning the chance to survival over 5 years after transplantation[1-3]. Most recipients need lifelong immunosuppression to prevent acute rejection and achieve ideal long-term outcomes[4]. However, the long-term side-effects caused by immunosuppressant usage, like opportunistic infection, malignant tumor, metabolic disorders and renal dysfunction have become the dominant obstacle to the long-term survival rates and life quality of LT recipients, especially for pediatric ones[5]. When matching by gender and age, LT recipients suffer a 2.4-fold higher risk of death and a 5.8-fold higher risk of premature death than the general population [6]. Therefore, strategies facilitating reduction or discontinuation of immunosuppressant are highly desirable.

Safely minimizing or discontinuing immunosuppressant without compromising allograft function could be an attractive strategy to improve the long-term post-LT survival[7,8]. The liver is considered a tolerogenic organ as LT recipients require less immunosuppressants and suffer lower risk of immune rejection when comparing with other solid organ recipients[9-11]. Anatomically, antigen-rich blood from the gastrointestinal tract flow through the intrahepatic sinusoids and scanned by antigen-presenting cells (APCs) and lymphocytes, while liver sinusoidal endothelial cells (LSECs) and hepatocytes act as scavenger cells contributing to the clearance of antigens[12-15]. Apoptosis of cytotoxic T lymphocyte (CTL) that induced by FasL and Programmed death ligand 1 (PD-L1) expressed by LSECs and hepatic stellate cells facilities the maintenance of the tolerogenic state[16,17]. Regulatory immune cells inside the liver like regulatory CD4+ T cell (Treg), Regulatory B cell (Breg) and regulatory dendritic cell (DCreg) also contribute to the development of tolerance by suppressing intrahepatic immune assault[18]. Traditionally, tolerance could be achieved through spontaneous, operational and induced ways. The first two means for tolerance were generally conducted in long-term follow-up recipients, while induced tolerance could be finished at early stage after transplantation, regardless of recipient's medical background, which makes it more applicable in clinical practices. Cellular strategy by infusion of *ex vivo* regulatory immune cell to create suppressive immune environment is the mainstream to achieve inducible tolerance. So far, many clinical and preclinical trials have been conducted to prove the efficacy of induced tolerance in LT recipients. Although promising preclinical and early-stage clinical results have proven the safety and feasibility of cellular therapy, its application in clinical practices requires more validation (Figure 1).

## TREGS AND THE INDUCTION OF TOLERANCE

Treg is a specialized subset of CD4 T cells characterized by the high expression of FoxP3 and interleukin-2 (IL-2) receptor CD25, and low expression of IL-7 receptor CD127[19]. Based on developmental origins, CD4+ Tregs could be divided into thymic Tregs (tTregs) and peripheral Tregs (pTregs). Functionally, tTregs primarily recognize self-antigens, whereas the pTreg could recognize "non-self" pathogens like infectious antigens or gastrointestinal commensal microbiota-derived antigens[20,21]. Tregs induce immune tolerance through a variety of pathways, including direct and indirect pathways. Currently, adoptive transfer of Tregs is becoming an attractive therapy to restore self-tolerance in autoimmune diseases and preventing occurrence of graft *vs* host disease (GVHD) after hematopoietic transplantation[20,22]. Valuable information has arisen from multiple clinical trials designed to test the safety and efficacy of Treg therapy in solid organ transplantation. Infusion of peripheral polyclonal Tregs in kidney transplantation recipients had proven the safety and feasibility of Treg therapy in solid organ transplantation recipients[23-25]. The first study to describe successful withdrawn of immunosuppressant following Treg therapy was reported by Todo *et al*[26] (UMIN-000015789), in which 7 out of 10 Living donor liver transplant recipients achieved tolerance[26]. However, less than 20% of the cell product in this study was defined as Tregs, which made it difficult to determine the precise immunoregulatory mechanisms involved. Then Sánchez-Fueyo *et al*[27] evaluated the safety and applicability of autologous polyclonal Treg adoptive

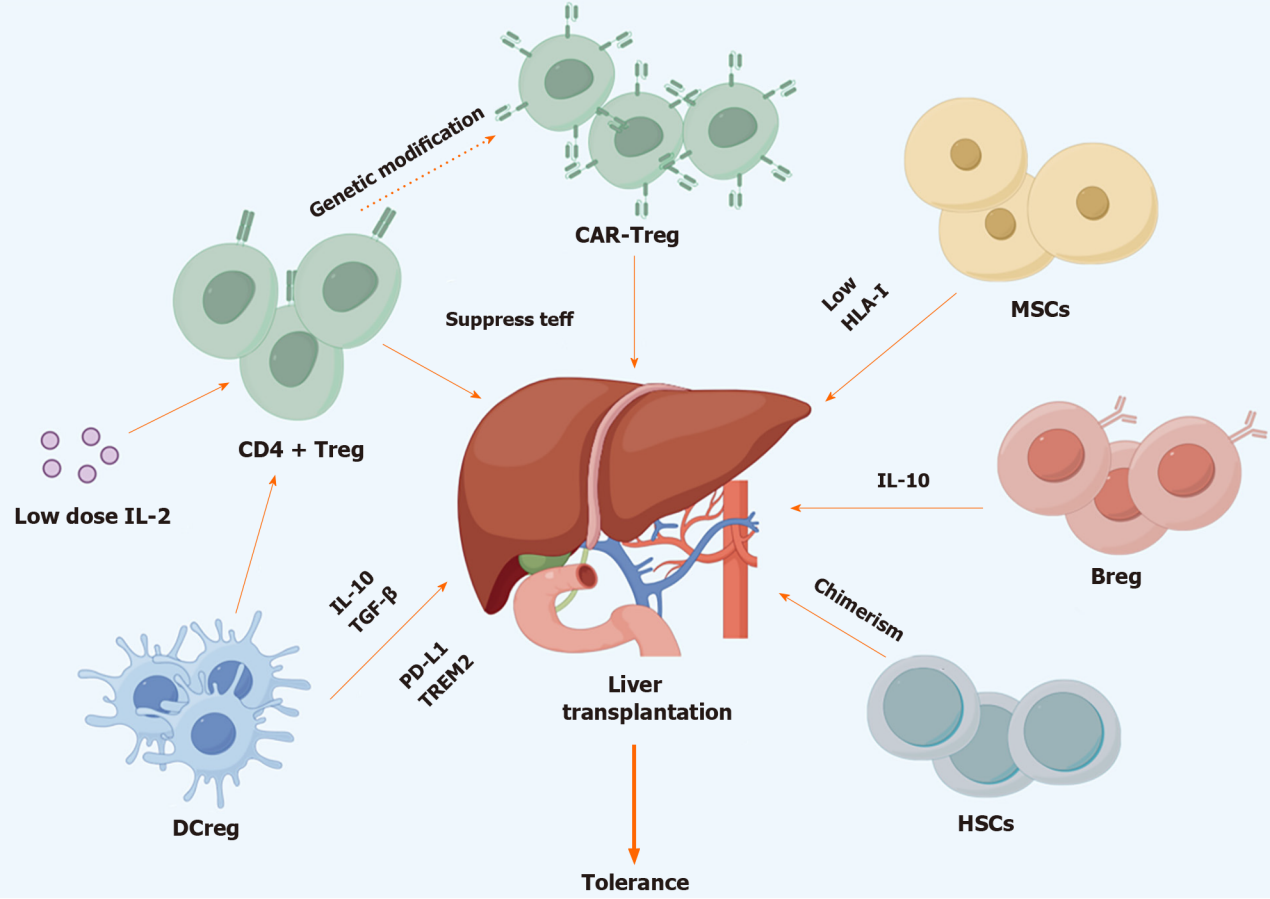


**Figure 1 Cellular strategies using regulatory immune cells to induce tolerance after liver transplantation.** Breg: Regulatory B cell; DCreg: Regulatory dendritic cell; HSCs: Hematopoietic stem cells; MSCs: Mesenchymal stromal cells; Treg: Regulatory CD4+ T cell; CAR: Chimeric antigen receptors; IL-2: Interleukin-2; HLA: Human leukocyte antigen; TGF-β: Transforming growth factor β; PD-L1: Programmed death ligand 1.

transfer in adult LT recipients through a phase I single-center clinical trial (ThRIL, NCT02166177)[27]. They found that Treg transfer was safe, transiently increased the amount of peripheral circulating Tregs and reduced T cell responses to donor antigens, which might facilitate the reduction or complete discontinuation of immunosuppression following LT. More recently, Tang *et al*[28] reported the results of a phase I/II trial (ARTEMIS, NCT02474199) of autologous donor alloantigen reactive Treg therapy in living donor liver transplant recipients. Four of five recipients who received sufficient infusion dosage encountered acute rejection during the process of immunosuppressant withdrawal[28]. Therefore, despite the capability of Tregs to ameliorate acute rejection in several preclinical studies, we are far from achieving induced post-LT tolerance in the clinic.

Expanding the circulating Tregs through cytokines treatment has also been tested. Since studies have suggested that Tregs have a reduced IL-2 receptor (IL-2R) signaling threshold than Teff cells, it has been hypothesized that the administration of low doses of IL-2 could preferentially activate Tregs and limit the activation of effector T cells[29,30]. In a murine skin transplantation model, IL-2 treatment with donor-specific Tregs infusion preferentially enhanced the proliferation of Tregs in skin allograft and draining lymph nodes, which prolonged skin allograft survival[31]. Lim *et al*[32] conducted the first clinical trial of using low-dose IL-2 to induce immune tolerance in adult LT recipients (NCT02949492). Although all participants achieved increased circulating Tregs after treatment, no expansion of donor-reactive Tregs or accumulation of intrahepatic Tregs was found, which was accompanied an interferon-γ dependent inflammatory response[32]. Reasons for the failure of IL-2 induced tolerance includes off-target effects of IL-2 to other immune cells, heterogeneity of IL-2 expanded Tregs and lack of intrahepatic infiltrated Tregs after treatment[33,34]. Therefore, IL-2 mutants or alternative induction approaches should be explored in the future.

Another approach to induce tolerance using Tregs is to generate antigen specific Treg cells by introducing synthetic chimeric antigen receptors (CARs) or engineered T cell receptors, enabling direct antigen recognition in the context of an antigen-major histocompatibility complex (MHC)-peptide complex[20]. In murine model, engineered CAR-Tregs with the ability to directly recognize allogeneic MHC class II molecules could facilitate the long-term acceptance of MHC-mismatched allograft[35]. Human CAR-Tregs targeting the human leukocyte antigen (HLA)-A2 could prevent HLA-A2-positive cells mediated xenogeneic GVHD in mouse models[36]. A multicenter phase I/II clinical trial aiming to evaluate the safety and tolerability of autologous anti-HLA-A2 CAR-Tregs in LT recipients (LIBERATE, NCT05234190) had been launched in Europe, while no further results had been reported so far. Since autologous CD4+ T cells and DCs played an important role in mediated posttransplant rejection, CAR-Treg targeting CD83, which was mainly expressed on alloreactive conventional CD4+ T cells and proinflammatory DCs had been proven to be efficient in preventing GVHD after

hematopoietic cell transplantation[37]. Another target protein for CAR-Tregs therapy is GAD65, which had been proved efficient to suppress CTLs in diabetes and islet transplantation mouse model[38]. However, since some studies of CAR effector T cells suggested that the density of the antigen recognized by the CAR must be high on the target cell to trigger activation, the efficiency of CAR-Tregs in the induction of tolerance still need more exploration[39].

## DENDRITIC CELLS AND TOLERANCE INDUCTION

Dendritic cells (DCs) are potent APCs linking the innate and adaptive immune process[40]. DCregs are characterized by reduced expression of MHC and co-stimulatory molecules (like CD80 and CD86), and increased level of death-inducing ligands (FasL) and co-inhibitory ligands (PD-L1)[4,41]. Functionally, DCregs are able to produce anti-inflammatory cytokines [IL-10 and Transforming growth factor β (TGF-β)] and impede T cell proliferation[42,43]. Unlike conventional DCs in secondary lymphoid tissue, intrahepatic DCs display tolerogenic properties. Intrahepatic DCs express comparatively low levels of Toll-like receptor 4, leading to limited adaptive immune response[44-46]. DCs express human leukocyte Ig-like receptor B family members result suppression of T cell responses[47]. Murine model indicated that Flt3 and DAP12 regulated liver myeloid DCs maturation and tolerance[46,48]. Meanwhile, donor-derived plasmacytoid DCs express high levels of DAP12, TREM2 and PD-L1 to attenuate graft-infiltrating effector T cell responses, enhance CD4+ Tregs function and promote spontaneous acceptance of allografts[49]. Therefore, application of tolerogenic DCs or DCregs could be an alternative approach to reach the goal of induced tolerance after LT.

The safety and feasibility of autologous DCreg therapy have been confirmed in autoimmune disorders, including rheumatoid arthritis, type I diabetes and Crohn's disease[50-52]. Many studies in murine transplantation model have confirmed the ability of donor derived DCs to function immunoregulatory properties and enhance organ allograft survival[53,54]. A clinically relevant nonhuman primate model also confirmed the safety and efficacy of donor derived DCs in prolonging MHC mis-matched renal allograft survival[55]. Angus W Thomson performed the first-in-human prospective study of donor-derived DCregs in LT recipients (NCT03164265), which proved the safety of DCreg therapy and changes of immune status after infusion[42]. However, no increase of tolerance rates in LT recipients has been observed so far[56]. One possible reason is the short-lived survival of donor DCreg after infusion, which may be killed by the NK cells. Meanwhile, the influence of donor derived DCreg to the immune status of the recipients is unclear. Even though circulating Treg/Teff ratio witness increase after DCreg infusion, whether the change is sufficient to induce tolerance is questionable. Therefore, although DCs are critical in the balance between allograft rejection and tolerance, extensive data from clinical trials and mechanism study are required before translating DCreg therapy into clinical practice in LT recipients.

## MESENCHYMAL STROMAL CELLS AND TOLERANCE INDUCTION

Mesenchymal stromal cells (MSCs) are nonhematopoietic multipotent and self-renewing cells with the ability to differentiate into mesodermal lineages like chondrocytes, adipocytes and osteocytes[57]. Surface marker profiles of MSCs include high expression of CD73, CD105 and CD90, and negative expression of CD45, CD34, and CD19[58]. Under normal conditions, MSCs express low levels of HLA-I molecules and do not express HLA-II nor co-stimulatory molecules, which renders MSCs immunoregulatory and anti-inflammatory properties[57,59]. Meanwhile, MSCs can be isolated from diverse tissues and are easy to cultivate, expand and store without losing clinical applicability *in vitro*[60,61]. In murine models, MSCs polarize both naïve and memory T cells toward Foxp3+ Treg phenotype and induce long-term graft acceptance[62-64]. Based on the preclinical results, lots of clinical trials have been conducted to study the therapeutical potentials of MSCs. Several pilot studies have proved that donor-derived bone marrow MSCs combined with a sparing dose of immunosuppressant dosage could maintain normal allograft function and don't increase the acute rejection occurrence in kidney transplantation recipients[65,66]. Yves Beguin performed the first human phase I clinical trial (NCT01429038) exploring the safety and tolerability of third-party MSCs infusion in LT recipients[67]. This study showed no toxicity, but a single MSC infusion was not sufficient to allow discontinuition of immunosuppression. Casiraghi *et al*[68] further revealed that MSCs infusion in LT recipients prior to transplantation was safe and could induce positive changes in peripheral immunoregulatory T and NK cells, but no tolerance data was reported[68]. The MYSTEP1 trial (NCT02957552) is the first clinical trial aiming to investigate the safety and feasibility of donor-derived NSCs in pediatric LT recipients, while no further data is available so far[69]. Pre-clinical studies in transplantation models exhibited a comparable capacity of autologous and allogeneic MSCs to induce Treg expansion and prolong allograft survival[70]. A single-center prospective clinical trial (NCT00658073) to inoculated living kidney transplantation recipients with bone marrow derived autologous MSCs revealed that autologous MSCs therapy resulted in lower incidence of acute rejection, decreased risk of opportunistic infection and better estimated renal function[71]. Modifications of MSCs like cytokine pretreatment, genetic modification or three-dimensional culture can improve the immunoregulatory capacity of MSCs and may be an effective approach to improve the regulatory capacity of MSCs under transplantation circumstance[72]. In rat LT model, infusion of TGF-β overexpressing or HO-1 transduced MSCs could induce a local immunosuppression in liver grafts, ameliorate the acute rejection and reduce the overall mortality[73,74]. However, no genetic modified MSCs have been applied in clinical trial so far. More detailed study to the molecular mechanism to the regulatory feature of MSCs is required before its clinical application (Table 1).

**Table 1 Clinical trials using cellular therapy to induce tolerance after liver transplantation**

| Ref. | Cellular products | Sample size/stage | Recipients | Status | Trial ID |
|---|---|---|---|---|---|
| Todo *et al*[26] | Donor derived Treg | 10/Phase I/II | Adult | 7/10 recipients reached tolerance | UMIN-000015789 |
| Sánchez-Fueyo *et al*[27] | Recipient derived polyclonal Treg | 6/Phase I | Adult | Safe for recipients, not test tolerance | NCT02166177 |
| Tang *et al*[28] | Recipient derived darTreg | 5/Phase I/II | Adult | 4/5 encountered acute rejection | NCT02474199 |
| Lim *et al*[32] | IL-2 infusion | 5/Phase I/II | Adult | All suffered rejection | NCT02949492 |
| Sánchez-Fueyo *et al*[91] | CAR-Treg targeting HLA-A2 | 18-70/Phase I/II | Adult | Recruiting | NCT05234190 |
| Tran *et al*[92] | Donor derived DCreg | 13/Phase I/II | Adult | Safe for recipients, no tolerance tested | NCT03164265 |
| Detry *et al*[67] | Third party MSCs | 10/Phase I/II | Adult | Safe for recipients, no tolerance achieved | NCT01429038 |
| Casiraghi *et al*[68] | Third party MSCs | 10/Phase I/II | Adult | Safe for recipients | NCT01429038 |

Treg: Regulatory CD4+ T cell; IL-2: Interleukin-2; CAR: Chimeric antigen receptors; HLA: Human leukocyte antigen; DCreg: Regulatory dendritic cell; MSCs: Mesenchymal stromal cells.

## OTHER CELLULAR STRATEGIES TO INDUCE TOLERANCE

Infusion of hematopoietic stem cells (HSCs) to create mixed chimerism could establish donor-specific tolerance and retain immunocompetence for primary immune responses[75,76]. Kawai *et al*[77] conducted the first successful application of mixed chimerism in tolerance induction in human kidney transplantation[77]. Four of five recipients who received combined bone marrow and kidney transplants from HLA single-haplotype mismatched living related donors and nonmyeloablative preparative regimen discontinued all immunosuppressive therapy with normal renal function. Patients with end stage renal disease and hematologic malignancies are thought as the most suitable candidates for combined bone marrow and kidney transplant[78]. The idea of hematopoietic chimerism to achieve graft tolerance has also been explored in LT recipients. Spontaneous complete hematopoietic chimerism could be found in deceased donor LT recipient even without HSCs transplant and tolerance was achieved[79]. Tryphonopoulos *et al*[80] reported that donor bone marrow cell infusion had no influence on the overall survival rates or tolerance of adult LT recipients[80]. Kim *et al*[81] and Hartleif *et al*[82] indicated that LT with myeloablative HSC transplant could establish full tolerance in both pediatric and adult recipients, but the life-threatening complication of GVHD couldn't be avoided[81-83]. Thus, the current dilemma of HSC therapy is that intense myeloablative or non-myeloablative conditioning therapy may not be tolerated by transplantation recipient, while lacking conditioning therapy could compromise the therapeutic efficiency of donor HSC infusion[80]. Therefore, careful selection of recipients might be the key to the safety and efficiency of HSCs therapy.

Bregs are immunosuppressive cells that express immune regulatory cytokines, like IL-10, TGF-β and IL-35, and support immunological tolerance[84]. In autoimmune disease mice model, the most widely investigated Breg population comprises the IL-10 producing B10 cells which could modulate T cell function[85]. It was found that B lymphocytes could interact with allo- and autoreactive effector cells, while selective manipulation of B cell function rather than depletion could be a promising approach to promote tolerance to allografts[86]. In murine heart and islet transplantation models, combined treatment with anti-CD45RB and anti-ICAM/LFA/TIM1 facilitated allograft acceptance *via* B-cell dependent mechanism[86,87]. A possible explanation is that B cells act as Treg inducing antigen presenting cells to promote Tregs function during this process. Single-cell RNA sequencing data of transplanted murine kidney revealed a shifting from a T cell-dominant to a B cell-rich population at 6 months after transplant with an increased Breg signature, implicating a key role of Bregs in the maintenance of allograft tolerance[88]. Analysis to stable renal transplantation recipients also revealed that B cells from tolerant patients had lower numbers of plasma cells and secreted more IL-10, which reduced production of proinflammatory cytokines and promoted transplantation tolerance[89,90]. However, so far, no clinical trial using Bregs to induce tolerance after transplantation have been conducted. One of the challenges is the lack of lineage marker for Bregs, which impedes the *in vitro* and *ex vivo* isolation and expansion of Bregs. Another problem is the unclear underlying mechanism of Bregs in the process of tolerance induction. Therefore, Breg induced tolerance has a long way to go before translation into clinical practice.

## CONCLUSION

Immune tolerance is one of the most promising approaches to avoid the long-term side-effects of immunosuppressants in LT recipients. Cellular therapy could be applied before and after transplantation, which could induce early tolerance. So far, many clinical trials have demonstrated the feasibility and safety of cellular therapies for autoimmune diseases,

hematopoietic stem cell transplantation and solid organ transplantation. However, most clinical results for cellular induced tolerance after LT are still very preliminary. The most obstacle is how to improve the efficiency of induced tolerance by cellular therapy. Detailed study to underlying mechanisms of immunoregulatory immune cells, genetic modification and optimal infusion dosage should be conducted in the future.

## FOOTNOTES

**Co-first authors:** Ai-Wei Zhou and Jing Jin.

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**Country/Territory of origin:** China

**ORCID number:** Yuan Liu 0000-0003-4033-1026.

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EDITORIAL

# Disease clearance in ulcerative colitis: A new therapeutic target for the future

Syed Adeel Hassan, Neeraj Kapur, Fahad Sheikh, Anam Fahad, Somia Jamal

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**Syed Adeel Hassan, Neeraj Kapur,** Division of Digestive Disease and Nutrition, University of Kentucky, Lexington, KY 40536, United States

**Fahad Sheikh,** Department of Pathology and Laboratory Medicine, Montefiore Medical Center, Albert Einstein College of Medicine, New York, NY 10461, United States

**Anam Fahad,** Division of Primary Care, Essen Healthcare, New York, NY 10457, United States

**Somia Jamal,** Department of Internal Medicine, Karachi Medical and Dental College, Karachi 74700, Sindh, Pakistan

**Corresponding author:** Syed Adeel Hassan, MBBS, MD, Research Scientist, Division of Digestive Disease and Nutrition, University of Kentucky, 800 Rose Street, Lexington, KY 40536, United States. doc.syed92@gmail.com

## Abstract

Advancements in murine modeling systems for ulcerative colitis have diversified our understanding of the pathophysiological factors involved in disease onset and progression. This has fueled the identification of molecular targets, resulting in a rapidly expanding therapeutic armamentarium. Subsequently, management strategies have evolved from symptomatic resolution to well-defined objective endpoints, including clinical remission, endoscopic remission and mucosal healing. While the incorporation of these assessment modalities has permitted targeted intervention in the context of a natural disease history and the prevention of complications, studies have consistently depicted discrepancies associated with ascertaining disease status through clinical and endoscopic measures. Current recommendations lack consideration of histological healing. The simultaneous achievement of clinical, endoscopic, and histologic remission has not been fully investigated. This has laid the groundwork for a novel therapeutic outcome termed disease clearance (DC). This article summarizes the concept of DC and its current evidence.

**Key Words:** Inflammatory bowel disease; Ulcerative colitis; Clinical remission; Endoscopic remission; Histological remission; Mucosal healing; Disease clearance

**Core Tip:** Clinical management of ulcerative colitis is guided by a combination of clinical and endoscopic measures, but histologic healing is undervalued. The current definition of disease remission is insufficient due to discrepancies in outcomes. Disease clearance (DC) is a novel emerging composite outcome defined as the simultaneous attainment of clinical, endoscopic and histologic remission. The risk of disease relapse, hospitalization and surgery is significantly lower in patients who achieve DC. It provides superior optimal disease control in the short term. Large prospective studies are needed to determine the cost effectiveness, risk-benefit ratio and impact on long-term outcomes.

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## INTRODUCTION

First described by Samuel Wilks in 1859, ulcerative colitis (UC) is a chronic, idiopathic relapsing inflammatory bowel disease (IBD) limited to the large intestine[1]. UC is characterized by chronic inflammation in the rectum and can progress continuously to the proximal colon[2,3]. The underlying etiology of this disease is considered multifactorial, with increasing focus on aberrant immune response, gut dysbiosis, a compromised gut epithelial barrier, genetic susceptibility and environmental factors[4]. Clinically, patients present with bloody diarrhea, urgency, abdominal pain and tenesmus[2]. Recent studies depict an increasing prevalence of UC, with an estimated 5 million cases globally[5]. In the United States, the epidemiological burden of UC is comparable with global trends, with an incidence and prevalence of 6.3 per 100000 and 378 per 100000 people, respectively[6]. Underlying long-term inflammation alters colonic anatomy and functionality, thus predisposing patients to several downstream sequalae. This subsequently impairs quality of life and increases the risk of disability and colorectal cancer (CRC)[7]. In addition to the significantly increasing disease burden, the progressive and debilitating nature of UC results in a significant economic burden owing to increased direct and indirect costs associated with health care utilization[8]. Pharmacoeconomic data from the Crohn's and Colitis Foundation of America estimated that the annual economic costs are between US$14.6 and US$31.6 billion[9].

In contrast to its counterpart Crohn's disease, UC has not been considered a progressive disease[10]. This perception has been rightly altered with the availability of evidence that suggests otherwise[10]. Proximal disease progression is observed in approximately 50% of patients with limited UC at diagnosis[10]. The risk of progression increases with disease duration, notably at 15%, 30% and 50% at 5 years, 10 years and 25 years, respectively[10-12]. An aggressive disease course can lead to colectomy in 10%-15% of patients[12]. Furthermore, disease progression predisposes patients to greater needs for biologics, as well as greater risk for extraintestinal manifestations, pseudopolyposis, anorectal dysfunction, gut dysmotility, surgeries and hospitalizations[13-15].

Due to the availability of only less potent drugs, the natural disease course has not been fully elucidated. Over the past few decades, advancements in murine modeling systems have yielded novel mechanisms of disease onset and progression[16]. This has fueled identification of a wide array of molecular targets, resulting in a rapidly expanding therapeutic armamentarium. The introduction of tumor necrosis factor inhibitors in 2005 set the tone for utilizing advanced therapies in UC[17]. However, their use is complicated by the abundance of serious adverse events, suboptimal response rates and loss of response[18]. Modern biologics and small molecules, such as anti-interleukins, anti-integrins, sphingosine-1-phosphate modulators, and Janus kinase inhibitors (JAKis), provide a cost-effective means of targeting natural disease history[19]. No significant difference in overall safety outcomes was observed between UC patients receiving JAKis and patients receiving other active treatments[20]. Therefore, the safety of JAKis can also be debated. The availability of myriad therapies has shifted therapeutic goals from symptomatic resolution to well-defined objective end points, clinical remission, endoscopic remission and mucosal healing[21,22].

Disease status ascertainment based on clinical and endoscopic outcomes is inadequate. To date, despite the availability of adequate evidence, the utility of histological healing remains limited[21,22]. Emerging evidence supports the impact of attaining simultaneous clinical, endoscopic and histological remission on disease outcomes. Herein, we discuss the emerging concept of disease clearance (DC) and the currently available evidence with a view to expanding its applicability in prospective high-profile research and its transition to clinical utility.

## TREAT TO TARGET: CURRENT GUIDELINES

DC incorporates components of target indices proposed in the selecting therapeutic targets in inflammatory bowel disease (STRIDE) program guidelines[21,22]. To better grasp the reasoning for DC, it is imperative to review current therapeutic target recommendations for UC. In 2015, the STRIDE committee added a new dimension to treatment aspirations with the introduction of treat-to-target (T2T) therapy[21]. Prior to the T2T concept, the primary aim of therapy was to achieve steroid-free disease remission based on the absence or presence of clinical symptoms[21]. However, this approach fails to alter disease progression or prevent long-term disease sequalae[21,23,24]. In recent decades, promising data have supported the use of more objective endpoints in clinical practice and trials. Achieving endoscopic remission or

mucosal healing was associated with improved long-term outcomes, such as steroid-free clinical remission; lower steroid utilization; and reduced rates of colectomy, dysplasia, CRC, disease relapse and hospitalization[21,25-30]. This finding supported the paradigm shift of treating beyond symptoms with a view to preventing structural damage and disability [21]. Furthermore, evidence of the effectiveness of T2T in treating other conditions, such as rheumatoid arthritis, diabetes and hypertension, supports its use in treating IBD[21,31-33]. Therapeutic adjustments were proposed on the basis of the achievement of predefined treatment goals with the aim of attenuating disease pathophysiology[21,34]. Subsequently, the importance of endoscopic assessment was outlined in the STRIDE[21]. Thus, the T2T strategy recommends the incorporation of a composite measure to ascertain disease status based on clinical remission/patient-reported outcomes (PROs) and endoscopic remission[21]. Clinical remission was defined as the resolution of rectal bleeding (RB) and normalization of stool frequency (SF)[21]. Clinical symptoms must be monitored every 3 months[21]. After adequate symptomatic control was achieved, follow-up every 6 months was considered adequate[21]. Endoscopic remission was defined as a Mayo endoscopic subscore of 0 or 1[21]. Endoscopic assessments were warranted every 3-6 months after the initiation of therapy[21]. When endoscopic evaluation is limited, resolution of inflammation should be ruled out by cross-sectional imaging[21]. A lack of evidence prevents the incorporation of histologic targets[21]. Finally, inflammatory biomarkers such as C-reactive protein (CRP) and fecal calprotectin (FC) were identified as adjunctive measures of inflammation rather than treatment targets[21].

Accumulating evidence and advancements in diagnostic modalities led to updated STRIDE guidelines[22,35]. In 2021, the STRIDE 2 guidelines incorporated time-dependent objective treatment targets ranging from short-term, intermediate-term and long-term goals of care[22]. The short-term target ensures that patients achieve a symptomatic response[21]. Intermediate goals include symptomatic remission, normalization of CRP levels and a decrease in FC to an acceptable range[21]. Approximately 15%-30% of patients fail to achieve a CRP response[36]. Therefore, the use of FC is preferred in biomarker assessments of inflammation[36]. The long-term treatment goals were endoscopic healing, normalization of quality of life and lack of disability[21]. Owing to superior disease outcomes, the endoscopic healing criteria were more stringent, with a Mayo endoscopic subscore of 0[22,30]. The low cost, ease of collection and lack of data from randomized controlled trials (RCTs) have led to treatment optimization driven by inflammatory biomarkers (CRP and FC)[22,37,38]. Despite the availability of evidence supporting the strong association of histologic healing (HH) with endoscopic healing and as a predictor of long-term outcomes, HH was endorsed only as an adjunctive target[22].

## DC IN UC

### *What is DC?*

DC is a novel emerging concept that has been adapted from dermatology[39]. DC is a composite measure defined as deep and comprehensive remission[40]. It encompasses the simultaneous attainment of clinical, endoscopic and histologic remission[40]. As a composite outcome, it holds the potential to improve treatment efficacy by increasing event rates and assessing all factors impacting disease activity[40]. Utilization of DC in psoriasis patients has yielded significant improvements in quality of life and disease control[41,42]. While it may represent the ultimate therapeutic target for psoriasis, DC was achieved in only 35.3% of patients[42]. DC has also demonstrated use in aiding therapeutic positioning in biologic drug efficacy comparator trials[43]. To avoid confusion in patients, DC should not be used synonymously with the term “cure”.

### *Why incorporate DC in UC?*

Current treatment goals devised by STRIDE committee utilize these endpoints individually at several predetermined targeted time points[21,22]. STRIDE 2 proposes focusing on the short to long term in a T2T manner[22]. Several discrepancies exist indicating that our current definition of disease remission is subpar. Despite achieving endoscopic healing, persistent RB and increased SF can be observed in 39% and 24%, respectively, of patients[44]. A subset of these persistent symptoms can be attributed to functional disorders, with irritable bowel syndrome being the most prevalent[45-48]. Chronic inflammation alters colonic physiology and anatomical integrity, resulting in abnormal colonic motility, a reduction in goblet cells, aberrant barrier function and sequalae of intestinal fibrosis[49-52]. The extent and location of these changes contribute to persistent PROs despite adequate disease control[44,53,54]. This increases therapeutic risks due to aggressive treatment strategies and unnecessary changes in therapy. On the other hand, there remains a risk of underlying endoscopic disease in 20%-50% of patients attaining symptomatic remission[44,55-57]. Therefore, the assessment of clinically asymptomatic patients must be supplemented by other objective measures of inflammation. Patients in clinical remission are less likely to seek medical attention, thus increasing their risk of developing sequalae related to unchecked smoldering inflammation[58]. Despite the use of modern therapeutics, 10%-30% of patients still require proctocolectomy for medically refractory disease[59,60]. The absolute risk of colectomy increases with each subsequent switch in therapy[60]. While response rates vary across drug classes, 30%-40% of patients fail to respond to initial therapy[61]. Patients primed with prior biologic exposure exhibit a stepwise reduction in response to subsequent therapies[61]. However, remission rates remain suboptimal, with 20%-30% of patients achieving disease remission in UC induction trials, indicating a perceived therapeutic ceiling[17,62-70].

The persistence of histologic activity despite endoscopic inactivity has been shown to increase the risk of disease progression, relapse and complications[71-73]. A discordance between histologic activity and quiescent macroscopic activity has been reported in > 30% of cases[74]. Compared with mucosal healing, histologic activity has shown superior performance as an independent predictor of clinical course[74-76]. Histologic inflammation has also been deemed an independent risk factor for the development of CRC[7]. A 3- to 5-fold increase in the risk for CRC has been observed in

patients with persistent histological activity[72]. The severity of histologic inflammation correlates with progression to advanced neoplasia[72]. Reversal of histologic disease has been shown to reduce the risk for CRC[77]. Achieving composite histologic and endoscopic improvement and remission correlate with PRO and reduced disability[78,79]. Despite being achievable in 55% of patients and growing evidence, histologic remission has not been formally designated as a therapeutic target[21,22,75]. In the majority of RCTs and regulatory trials, histological outcomes have been positioned as an exploratory or additional endpoint rather than a mandatory endpoint. Furthermore, the availability of multiple validated histological scoring systems, uniform endoscopic disease distribution and excellent predictive ability of inflammatory biomarkers for HH will facilitate the application of DC as a therapeutic outcome in UC[80-82]. This has given rise to the concept of deeper disease control by incorporating histologic activity as a mandatory endpoint. The data here support the hypothesis of total deep remission when combined with clinical, endoscopic and histological outcomes.

### *Consensus definitions of DC by the International Organization for the Study of IBD*

In 2023, the first ever standardized guidelines defining DC were published by the International Organization for the Study of Inflammatory Bowel Diseases (IOIBD)[40]. It is expected that these guidelines will help standardize its use. Acknowledgment of the prognostic importance of histologic outcomes led to its incorporation as an official therapeutic endpoint[40]. The definition finalized by the IOIBD includes clinical, histologic and endoscopic remission of disease[40]. This culminates in all major time-dependent objective measures proposed in STRIDE 2. The expert consensus further delved deep to closely define each desired therapeutic target in line with the best available evidence. Clinical remission was defined as the total absence of clinical symptoms with a partial Mayo score of 0[40]. A Mayo endoscopic score of 0 and a Nancy histologic score of 0 define endoscopic remission and histologic remission, respectively[40].

### *Evidence supporting DC in UC as a therapeutic target*

In a multicenter retrospective study, D'Amico *et al*[83] evaluated the impact of DC on long-term outcomes in UC patients. DC was defined as simultaneous clinical (partial Mayo score ≤ 2), endoscopic (Mayo endoscopic score 0) or histological remission (Nancy index 0)[83]. Adult UC patients with an endohistological evaluation within 16 wk postinduction and at least 12 months of follow-up were included. The median follow-up time was 24 months[83]. Of the 494 patients in the study, 109 (22%) achieved DC[83]. Patients who achieved DC had shorter disease durations (5 years *vs* 9 years, $P < 0.001$)[83]. Significantly lower rates of negative outcomes such as hospitalization (5.5% *vs* 23.1%, $P < 0.001$) and surgery (1.8% *vs* 10.9%, $P = 0.003$) were noted in patients who underwent DC[83]. When reanalyzed using more stringent criteria (Mayo endoscopic score 0, Nancy index 0, normal SF and absence of RB), 19.8% of the patients met the criteria for DC[83]. The rates of hospitalization (22.7% *vs* 5.1%, $P = 0.003$) and surgery (10.9% *vs* 1.0%, $P = 0.012$) were greater in patients without DC[83]. Taken together, attaining DC within 16 wk posttherapeutic induction significantly lowers health care expenditure, the risk of hospitalization and surgery (Figure 1). This underlines the importance of initiating early treatment. Furthermore, this finding indicates the need for developing more efficient drugs[84].

Andronic and Toader[85] conducted an analysis of 79 UC patients[85]. For the purposes of this study, DC was defined as clinical (partial Mayo score ≤ 2), endoscopic (endoscopic Mayo score ≤ 1) or histologic remission (nancy index 0)[85]. At the initial time points, patients were divided into two subgroups. Groups 1 and 2 were deemed to have DC ($n = 35$) and without DC ($n = 44$), respectively[85]. Patients in both groups were followed for 12 months. Patients who achieved DC (Group 1) did not experience disease complications or required surgery (0% *vs* 31.8%, $P = 0.03$, OR = 23.1)[85]. The rate of hospitalization was significantly lower in Group 1 than in Group 2 (8.57% *vs* 54.54%, $P = 0.002$, OR = 0.57, RR = 0.224)[85].

Nascimento *et al*[86] conducted a retrospective analysis of 56 patients with UC and DC at baseline[86]. DC was defined as clinical (partial Mayo score ≤ 2), endoscopic (endoscopic Mayo score ≤ 1) or histologic remission (chronic inactive/quiescent colitis)[86]. During the 3-year follow-up, none of the patients with DC required surgery, and only one was hospitalized[86]. The overall probability of maintaining remission was 76% at 3 years[86].

A natural question arises whether the achievement of such a stringent endpoint is possible. Kruis *et al*[87] conducted a post hoc analysis of 4 phase 3 clinical trials[87]. The data were pooled from 860 UC patients to determine the percentage of DCs induced by different doses of mesalazine[87]. Overall, 20% achieved DC, 13% received 1.5 g/day, 21.8% received 3 g/day and 18.9% received 4.5/day[87]. Furthermore, the rates of DC were consistent across disease activities in a dose-dependent manner[87]. According to a post hoc analysis of the VARSITY comparator trial, at week 52, DC was noted in a greater percentage of patients receiving vedolizumab than in those receiving adalimumab (29.2% *vs* 16.3%)[88]. The data here suggest that DC is an achievable target in clinical practice, for which the likelihood of reaching this stringent outcome is 20%-29%.

## FUTURE AVENUES AND UTILITY IN CLINICAL LANDSCAPE

While still in its infancy, it is expected that the definition of DC will evolve to include ongoing or upcoming results[40]. Emerging evidence supports the correlation of rectal muscle remodeling (rectal compliance) with histologic normalization and impact on quality of life[89]. Newer measures of quality of life, such as fecal urgency, are also under investigation [40]. Future avenues must assess superiority with differences in long-term outcomes with DC *vs* histological remission alone. Currently, the ongoing VERDICT trial aims to determine whether DC alone is superior to steroid-free symptomatic remission or steroid-free endoscopic remission combined with clinical remission[90]. To aid in the transition to routine clinical care, DC should be considered a secondary endpoint in RCTs[40]. Current evidence of DC is mainly driven by retrospective and post hoc analyses. Most current studies were conducted prior to the release of standardized DC guidelines. Therefore, some studies utilize different definitions of clinical remission. To accurately assess the impact of

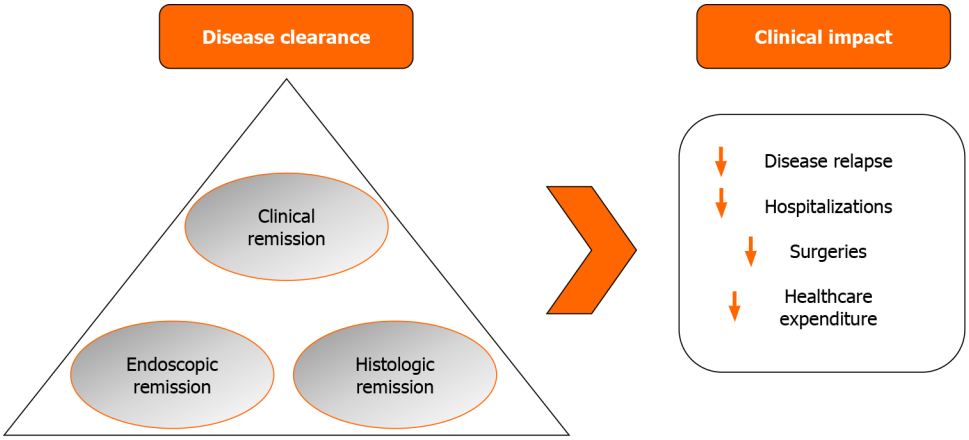


**Figure 1 Defining disease clearance in ulcerative colitis and its perceived impact on clinical outcomes.**

achieving DC on long-term outcomes, future studies must incorporate a uniform definition of DC. In addition, additional prospective studies with predefined objectives must be conducted. While patients who achieved DC were shown to have a lower baseline inflammatory burden, future studies must assess the role of inflammatory biomarkers. Given that achieving DC might be considered a difficult task, developing predictors of DC is important. The likelihood of achieving DC may be increased by dual therapy and by discovering biomarkers of drug response. Evidence pertaining to the value of dual therapy remains limited, with few prospective large-scale studies conducted to date[91]. Therefore, the role of dual therapy in inducing DC remains unknown. The impact of DC on dire sequalae such as dysplasia and CRC must also be ascertained. Transcriptional signatures specific to disease remission have also been delineated[40]. These include increased expression of genes regulating o-glycosylation and GAP junction trafficking and decreased expression of toll-like receptors[40,92]. This paves the way for the addition of molecular remission as an endpoint in DC with a direction for developing reliable molecular predictors of disease outcomes.

## CONCLUSION

DC is a novel therapeutic outcome in UC patients and has the potential to provide superior disease control and reduce the risk of long-term complications. Prospective studies are necessary to ascertain the cost effectiveness, risk-benefit ratio and impact on long-term outcomes.

## FOOTNOTES

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**Country/Territory of origin:** United States

**ORCID number:** Syed Adeel Hassan 0000-0002-8484-0319; Neeraj Kapur 0000-0001-6746-3331; Fahad Sheikh 0000-0001-9380-0661; Anam Fahad 0009-0004-8951-6480; Somia Jamal 0009-0008-1796-8903.

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EDITORIAL

# Risk factors for lymph node metastasis in superficial esophageal squamous cell carcinoma

Yan-Bo Yu

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**Yan-Bo Yu,** Department of Gastroenterology, Laboratory of Translational Gastroenterology, Qilu Hospital, Shandong University, Jinan 250012, Shandong Province, China

**Corresponding author:** Yan-Bo Yu, MD, PhD, Professor, Doctor, Department of Gastroenterology, Laboratory of Translational Gastroenterology, Qilu Hospital, Shandong University, No. 107 Wenhuaxi Road, Jinan 250012, Shandong Province, China.
yuyanbo2000@126.com

## Abstract

In this editorial, we comment on the article by Wang *et al* published in the recent issue of the *World Journal of Gastroenterology* in 2023. We focused on identifying risk factors for lymph node metastasis (LNM) in superficial esophageal squamous cell carcinoma (SESCC) patients and how to construct a simple and reliable clinical prediction model to assess the risk of LNM in SESCC patients, thereby helping to guide the selection of an appropriate treatment plan. The current standard treatment for SESCC is radical esophagectomy with lymph node dissection. However, esophagectomy is associated with considerable morbidity and mortality. Endoscopic resection (ER) offers a safer and less invasive alternative to surgical resection and can enable the patient's quality of life to be maintained while providing a satisfactory outcome. However, since ER is a localized treatment that does not allow for lymph node dissection, the risk of LNM in SESCC limits the effectiveness of ER. Understanding LNM status can aid in determining whether patients with SESCC can be cured by ER without the need for additional esophagectomy. Previous studies have shown that tumor size, macroscopic type of tumor, degree of differentiation, depth of tumor invasion, and lymphovascular invasion are factors associated with LNM in patients with SESCC. In addition, tumor budding is commonly associated with LNM, recurrence, and distant metastasis, but this topic has been less covered in previous studies. By comprehensively evaluating the above risk factors for LNM, useful evidence can be obtained for doctors to select appropriate treatments for SESCC patients.

**Key Words:** Superficial esophageal squamous cell carcinoma; Endoscopic resection; Lymph node metastasis; Risk factors; Tumor budding; Predictive model

**Core Tip:** Endoscopic resection is a routine treatment for superficial esophageal squamous cell carcinoma, but the risk of lymph node metastasis (LNM) limits its application to some extent. Tumor size, invasion depth, tumor differentiation, tumor infiltrative growth pattern, tumor budding, and lymphovascular invasion were shown to be significantly correlated with LNM.

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## INTRODUCTION

Esophageal cancer, the ninth most prevalent cancer and the sixth leading cause of cancer death worldwide, represents an important health concern that must be taken seriously. The overall 5-year survival rate is less than 15%. In 2020 alone, approximately 544000 deaths were attributed to esophageal cancer[1]. Esophageal squamous cell carcinoma (ESCC) is the most common subtype of esophageal cancer, accounting for 90% of esophageal cancers in Asia, Eastern Europe and Africa[2].

Malignant tumors localized to the mucosa or submucosa, with or without lymph node metastasis (LNM), are referred to as superficial esophageal squamous cell carcinoma (SESCC)[3,4]. The invasion of SESCC is limited to the mucosa and submucosa, causing no symptoms and posing challenges for early diagnosis of these patients. Currently, the use of new endoscopic techniques, such as magnifying endoscopy and narrow-band imaging endoscopy, and increased health awareness have significantly increased the detection rate of SESCC[5]. With detection at an early stage, timely and appropriate intervention usually results in a favorable prognosis.

The standard treatment for SESCC is radical esophagectomy with lymph node dissection. However, esophagectomy is not indicated for patients of advanced age or with multiple comorbidities due to the risk of complications and a significant reduction in quality of life postsurgery[6,7]. Endoscopic resection (ER) is a safer and less invasive alternative to surgical resection, which can maintain a patient's quality of life while achieving a satisfactory outcome. When the tumor is limited to the mucosa or submucosa, survival can reach 90% after endoscopic or surgical treatment[8]. The inability to perform lymph node dissection is the limitation associated with ER. Notably, prior research has demonstrated the importance of LNM in the unfavorable prognosis of SESCC[9,10] since a significantly lower 5-year survival rate has been observed in LNM-positive patients than in LNM-negative patients[11]. Even SESCC has the potential for LNM due to the abundance of the lymphocapillary plexus in the mucosa and submucosa of the lamina propria of the esophagus[12].

Because the probability of LNM increases proportionally with invasion depth, most current indications for ER in ESCC patients are formulated based on the depth of tumor invasion. The LNM rates were reported as follows: T1a-EP (epithelium) or T1a-LPM (lamina propria mucosa), 0.0%-3.3%; T1a-MM (muscularis mucosa) or T1b-SM1 (upper third of the submucosal layer), 0.0%-26.5%; and T1b-SM2, 22%-61%[13]. Generally, among ER, esophagectomy, and chemoradiotherapy, the treatment strategies for patients in the categories of T1a-MM and T1b-SM1 are regarded as borderline. According to the guidelines for esophageal cancer in Japan[14,15], clinical T1a-MM and T1b-SM1 SESCC are relative indications for ER, and additional treatment after ER is recommended for SESCC patients with lymphovascular invasion (LVI) or submucosal invasion. However, even if ER is performed, if the histopathological diagnosis is tumor depth [pT1a-MM, and LVI (+) or pT1b-SM], radical resection or additional treatment such as chemoradiotherapy is recommended due to considering the risk of LNM. The European Society of Gastrointestinal Endoscopy Guideline[16] suggests that endoscopic submucosal dissection might be considered for ESCC patients with noncircumferential clinical staging of T1a-MM/T1b-SM.

The predictors of LNM in SESCC patients can be assessed to determine whether they are likely to be cured by ER without additional esophagectomy or lymphadenectomy. Additionally, the clinicopathological risk factors associated with LNM in SESCC are still incompletely understood. Some imaging methods, such as endoscopic ultrasonography and CT, can be used to detect LNM in ESCC but have low accuracy, especially for T1 tumors[17,18]. In these cases, ER offers more precise staging in addition to therapeutic benefits[18].

## RISK FACTORS FOR LNM IN SESCC

Previous studies have shown that tumor size, macroscopic type of tumor, degree of differentiation, depth of tumor invasion, and LVI are factors associated with LNM in patients with SESCC[8]. In the recent issue of the *World Journal of Gastroenterology*, Wang *et al*[19] published an interesting paper titled "Risk factors and a predictive nomogram for LNM in superficial esophageal squamous cell carcinoma." This study developed a useful nomogram model to predict LNM risk in superficial ESCC patients. In this retrospective study, 474 SESCC patients who underwent esophagectomy at West China Hospital of Sichuan University from January 1, 2009 to January 31, 2016, were enrolled in the final analysis. Of those, 90 of the 474 (16.48%) patients had LNM, and the LNM rate was 3.29% (5/152) for T1a tumors and 26.40% (85/322) for T1b tumors. Variables such as tumor size, invasion depth, tumor differentiation, tumor infiltrative growth (INF)

pattern, tumor budding (TB), and LVI were significantly associated with LNM according to univariate analysis. Multivariate logistic regression analysis also showed that tumor size, invasion depth, tumor differentiation, the INF (tumor infiltrative growth) pattern, TB, and LVI were independent risk factors for LNM. The ROC curve showed that this nomogram had good predictive performance in both the training set and the validation set, with AUCs of 0.789 [95% confidence interval (95%CI): 0.737-0.841] and 0.827 (95%CI: 0.755-0.899), respectively.

In several studies, scholars have proposed predictive models for LNM in patients with superficial ESCC, but these models have several limitations[20,21]. As the invasion depth increases, the probability of LNM in SESCC increases proportionally. A limitation of some studies is that no further stratification of the submucosa was performed. The nomogram established by Wang *et al*[19] involved tumor categorization into 3 grades by depth of infiltration: MM, SM1, and SM2 and above. An invasion depth deeper than SM1 [odd ratio (OR): 15.517, 95%CI: 4.707-51.158] was an independent risk factor for LNM.

Wang *et al*[19] also incorporated TB into a prediction model, which has rarely been addressed in previous studies. TB is a morphological phenomenon of diffuse mucous infiltrative growth, characterized by the presence of isolated cells or clusters of tumor cells (up to 5 cells) scattered in the stroma at a variable distance from the invasive front of the tumor [22]. These cells detach from the tumor mass and migrate into the adjacent stroma, representing the first step toward invasive growth followed by metastasis. The presence of TB is commonly associated with a more aggressive cancer phenotype and is correlated with LNM, recurrence, and distant metastasis and thus poor survival. One hypothesis suggests that TB mimics epithelialmesenchymal transition, a process in which cells change from an epithelial phenotype expressing E-calmodulin and cytokeratin to a mesenchymal phenotype expressing vimentin and N-cadherin[23]. When cancer cells acquire a mesenchymal phenotype, cell polarity and intercellular adhesion are lost, leading to invasion and metastasis[24]. However, there are also hypotheses that the mechanism of TB is not related to epithelialmesenchymal transition[25,26]. Although underlying the mechanism is unclear, TB can be identified as a histopathological predictor of LNM or poor prognosis in gastrointestinal carcinoma including esophageal adenocarcinoma, ESCC, lung squamous cell carcinoma, and cervical cancer[27]. However, only a few studies have focused on TB and its importance in the prognosis of ESCC. There is no gold standard for determining the threshold value of TB in patients with SESCC.

In the study by Mitobe *et al*[28], univariate analysis showed that TB in SESCC was significantly associated with LNM. However, this study failed to show that TB was an independent risk factor for LNM. Min *et al*[20] reported that the presence of TB had borderline importance for LNM prediction. Fuchinoue *et al*[29] showed that the cutoff values for high-grade TB evaluated using hematoxylin-eosin (HE) staining or immunohistochemistry (IHC) were 2 and 11, respectively. High-grade TB, as evaluated using HE staining ($P$ = 0.007) and IHC ($P \leq 0.001$), was significantly correlated with LNM. For tumors with pT1a-MM to pT1b-SM1, high-grade TB evaluated using IHC was correlated with LNM ($P$ = 0.050). Li *et al*[30] found that TB according to a three-tiered grading system (low-TB, 0-4; middle-TB, 5-15; high-TB, ≥ 16) was an excellent prognostic indicator for LNM and survival based on IHC staining using a 20 × objective lens. Wang *et al*[19] categorized TB into three types based on HE staining: No budding, low-grade TB (1 to 4 budding foci at a 20 × objective lens), and high-grade TB (≥ 5 budding foci at a 20 × objective lens). The study showed that high-grade TB (OR: 3.905, 95%CI: 1.387-10.995) was positively correlated with LNM risk in SESCC patients. Furthermore, unlike previous studies, multifactorial regression analysis in this study showed that TB was an independent risk factor for LNM, helping to promote the use of tumor outgrowth in SESCC pathology diagnosis and advance its inclusion in routine pathology reporting.

## CONCLUSION

In summary, ER is known to be a routine treatment for SESCC, and considering the impact of LNM on patient prognosis, it is crucial to explore the predictors of LNM before ER in patients with SESCC. In addition, in patients with ER-treated SESCC, medical practitioners must assess the risk of LNM and thus the need for further esophagectomy based on post-ER pathological diagnosis. As a result, a practical decision-making tool built on multifactor analysis for assessing LNM risk is essential.

## FOOTNOTES

**Author contributions:** Yu YB designed the overall concept and outline of the manuscript and contributed to the writing, and editing the manuscript.

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**Country/Territory of origin:** China

**ORCID number:** Yan-Bo Yu 0000-0003-2995-3270.

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*REVIEW*

# Molecular insights into clinical trials for immune checkpoint inhibitors in colorectal cancer: Unravelling challenges and future directions

Samantha Sharma, Naresh Singh, Anita Ahmed Turk, Isabella Wan, Akshay Guttikonda, Julia Lily Dong, Xinna Zhang, Mateusz Opyrchal

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**Samantha Sharma, Naresh Singh, Isabella Wan, Akshay Guttikonda, Julia Lily Dong, Xinna Zhang,** Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Anita Ahmed Turk, Mateusz Opyrchal,** Division of Hematology/Oncology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Xinna Zhang, Mateusz Opyrchal,** Melvin and Bren Simon Comprehensive Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Corresponding author:** Samantha Sharma, PhD, Assistant Professor, Department of Medical and Molecular Genetics, Indiana University School of Medicine, 980 W Walnut St, R3.C260, Indianapolis, IN 46202, United States. samshar@iu.edu

## Abstract

Colorectal cancer (CRC) is a complex disease with diverse etiologies and clinical outcomes. Despite considerable progress in development of CRC therapeutics, challenges remain regarding the diagnosis and management of advanced stage metastatic CRC (mCRC). In particular, the five-year survival rate is very low since mCRC is currently rarely curable. Over the past decade, cancer treatment has significantly improved with the introduction of cancer immunotherapies, specifically immune checkpoint inhibitors. Therapies aimed at blocking immune checkpoints such as PD-1, PD-L1, and CTLA-4 target inhibitory pathways of the immune system, and thereby enhance anti-tumor immunity. These therapies thus have shown promising results in many clinical trials alone or in combination. The efficacy and safety of immunotherapy, either alone or in combination with CRC, have been investigated in several clinical trials. Clinical trials, including KEYNOTE-164 and CheckMate 142, have led to Food and Drug Administration approval of the PD-1 inhibitors pembrolizumab and nivolumab, respectively, for the treatment of patients with unresectable or metastatic microsatellite instability-high or deficient mismatch repair CRC. Unfortunately, these drugs benefit only a small percentage of patients, with the benefits of immunotherapy remaining elusive for the vast majority of CRC patients. To this end, primary and secondary resistance to immunotherapy remains a significant issue, and further research is necessary to optimize the use of immunotherapy in CRC and identify biomarkers

to predict the response. This review provides a comprehensive overview of the clinical trials involving immune checkpoint inhibitors in CRC. The underlying rationale, challenges faced, and potential future steps to improve the prognosis and enhance the likelihood of successful trials in this field are discussed.

**Key Words:** Colorectal cancer; Immune checkpoint inhibitors; Clinical trials; Immunotherapy; Microsatellite instability; Microsatellite stability; DNA mismatch repair



**Core Tip:** Colorectal cancer (CRC) often eludes early detection, limiting the efficacy of existing chemotherapy and targeted therapies. This article delves into the realm of immune checkpoint inhibitors in CRC, dissecting their mechanisms and outcomes through a comprehensive review of clinical trials. It sheds light on the underlying rationale, challenges faced, and potential strategies to improve prognosis and trial success in this critical domain. Notably, while microsatellite instability-high CRC exhibits heightened responsiveness to checkpoint inhibitors, the article underscores potential breakthroughs in treating microsatellite stable CRC-the predominant cases-providing insights into bettering prognosis and trial outcomes in CRC treatment.

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## INTRODUCTION

Colorectal cancer (CRC) is a prevalent malignancy recognized worldwide for its intricate pathogenesis, diverse etiologies, and clinical outcomes[1,2]. Approximately 147950 new cases are expected to be diagnosed in 2023, along with an estimated number of 53200 deaths due to CRC[3]. Moreover, the incidence of early onset CRC is increasing as well[4]. CRC arises from the malignant transformation of epithelial cells lining the colon or rectum. The development of CRC is influenced by a multitude of risk factors, including advanced age, dietary choices, obesity, and inflammatory bowel disease[5-7]. The molecular pathogenesis of CRC is complex, with genetic and epigenetic alterations that drive tumorigenesis and contribute to disease progression[8-12]. These alterations intricately disrupt essential signaling pathways, such as WNT/β-catenin pathway, KRAS/BRAF/MEK/ERK pathway, and PI3K/AKT/mTOR pathway governing critical cellular processes, including cell proliferation, differentiation, and survival[8,9,13-15].

Currently, several approaches are employed for CRC treatment, including surgical procedures, chemotherapy, radiation therapy, targeted therapy, and immunotherapy[16]. However, following preliminary diagnosis, the 5-year survival rate of CRC patients is 65.0%, which significantly decreases to approximately 13% for metastatic CRC (mCRC)[17]. Treatment of advanced or mCRC is hindered by several challenges. Treatment options are particularly limited for patients who have exhausted multiple lines of treatment. Additionally, CRC tumors can develop resistance to chemotherapy, diminishing treatment efficacy over time[18,19]. The toxicity associated with chemotherapy and targeted therapies further complicates treatment and affects patients' quality of life. mCRC also has poor prognosis. Tumor heterogeneity adds another layer of complexity, contributing to treatment resistance and variability in patient responses[20,21]. To this end, immunotherapy targeting immune checkpoints such as PD-1/PD-L1 axis and CTLA-4 shows promise in treating advanced CRC, particularly in CRC tumors with microsatellite instability-high (MSI-H) or deficient mismatch repair (dMMR)[22]. Combination therapies, involving immunotherapy with chemotherapy, targeted therapies, and other immunomodulators, further offer the potential of synergistic effects and enhanced treatment efficacy[23,24]. Ongoing research efforts on predictive biomarkers, such as tumor mutational burden (TMB) and immune cell infiltration patterns, aim to identify patients most likely to benefit from immunotherapy[25,26]. Hence, immunotherapy holds promise as a transformative approach for the management of advanced or mCRC with durable responses and improved patient outcomes.

Immunotherapy using immune checkpoint inhibitions (ICIs) has recently emerged as a promising therapeutic approach for various cancers, including CRC. Understanding the immune infiltration patterns in CRC patients with microsatellite-stable (MSS) *vs* microsatellite-instability (MSI) phenotype is crucial for developing immunotherapeutic strategies. While MSI-H tumors may benefit from immunotherapy due to their higher immune infiltration and mutational load, MSS tumors may still require alternative or combination approaches to enhance the antitumor immune response and improve treatment outcomes[27,28]. To this end, ongoing research efforts aim to unravel the complexities of immune infiltration in different CRC subtypes to guide the development of more effective and personalized therapeutic interventions. Initial studies conducted between 2010 and 2013 showed limited clinical activity of ICIs in patients who were not selected based on specific biomarkers or treatment history[29-33]. Eventually, several promising findings have led to the approval of ICIs for MSI-H or dMMR CRC. Nonetheless, a low response to immunotherapy remains a

significant challenge in the treatment of MSS or proficient MMR (pMMR) CRC, highlighting the need for further research to enhance effectiveness and identify biomarkers to improve treatment outcomes[34,35]. Newer immunotherapeutic approaches have been investigated for CRC treatment, including cancer vaccines, adoptive T-cell therapy, and oncolytic viruses[36,37]. These approaches aim to stimulate the immune response against cancer cells by various means, including inducing antigen-specific T cell responses, genetically modifying T-cells to recognize and attack cancer cells, and using viruses to selectively target and destroy cancer cells[38,39]. A multitude of clinical trials, spanning both ongoing and concluding studies, have been conducted to explore the efficacy and safety of diverse drugs and combination therapies for CRC treatment. This review provides insights into the current landscape, challenges, and potential advancements in this field. CRC clinical trials involving ICIs and their mechanistic actions are outlined, treatment strategies and the future trajectory of CRC therapeutics are discussed.

## MOLECULAR INSIGHTS AND THERAPEUTIC PROGRESS IN CRC

Molecular characterization of CRC has identified two major subtypes, MSS and MSI CRC that account for approximately 85% and 15% of all CRC cases, respectively[40-42]. Clinical and pathological features of MSS CRC differ from those of MSI CRC[2]. Specifically, MSS CRC is typically associated with older age, male sex, and distal colon location, whereas MSI CRC is associated with younger age, female sex, proximal colon location, and better prognosis[41]. Furthermore, the MMR status and CRC are intricately linked due to their role in maintaining genome integrity and preventing the accumulation of mutations that can lead to cancer[43,44]. MSI-H CRC tends to have a dMMR status, a higher mutational load, and a distinct molecular profile compared to MSS CRC, which has a pMMR[43-45]. In particular, MSI and MMR status are predictive biomarkers for response to ICIs therapy[34,42]. The consensus molecular subtype (CMS) classification system divides CRC into four distinct subtypes based on gene expression profiles: CMS1 (immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal)[46]. Each CMS subtype has a distinct molecular signature and clinical phenotype. The system thus offers a clear biological understanding and serves as a foundation for future clinical stratification and targeted interventions based on specific subtypes.

Over the years, substantial progress has been achieved in the development of CRC therapeutics, resulting in enhanced survival rates attributed to advancements in primary and adjuvant treatment modalities[47]. Notably, the inclusion of chemotherapy as a neoadjuvant or adjuvant intervention has emerged as a strategic approach aimed at mitigating the tumor burden, reduction, and stabilization[48-51]. Chemotherapy maintains its pivotal status in current treatment strategies. However, the utility of chemotherapy is curtailed by a restricted therapeutic range, significant adverse reactions, and the frequent occurrence of acquired resistance[52]. Several chemotherapy agents, radiotherapies, and other physical forces also induce destruction of cells and tissues, leading to death of immune cells and subsequently enhancing therapeutic outcomes[53-56]. Immunotherapy with checkpoint inhibitors has provided a significant improvement in cancer treatment, demonstrating high efficacy and manageable side effects in various tumor types[57-62]. However, the success of immunotherapy in CRC patients remains limited, with only a small subset of cases characterized by MSI-H or dMMR benefiting from treatment[63,64]. Thus, despite over 50 decades of research on immunotherapy for CRC treatment and major advancements, significant challenges remain in the diagnosis and management of CRC, particularly in the context of advanced or metastatic disease[27] (Figure 1).

Immuno-oncology is an emerging field of cancer treatment that involves harnessing the patient's immune system to recognize and eliminate cancer cells[64]. Immunotherapy can potentially improve treatment outcomes for patients with a wide range of malignancies[38]. Immunotherapy is often considered more beneficial than chemotherapy due to its ability to induce durable immune responses. Unlike chemotherapy, which primarily induces short-term cytotoxic effects on cancer cells and eliminates immunosuppressive cells[65-67], immunotherapy activates the immune system, particularly cytotoxic T-cells, to recognize and target cancer cells[38]. Consequently, immunotherapy offers the potential for sustained protection against cancer by maintaining an immunological "memory" that can detect and eliminate cancer cells in case of re-encounter[68,69]. Immunotherapy for cancer has thus brought about revolutionary transformations in the field of oncology, extending the survival of individuals diagnosed with aggressive life-threatening malignancies[57-61]. CRC patients with MSI-H or dMMR status show higher mutation rates, more neoantigens, and increased tumor-infiltrating lymphocytes (TILs), particularly cytotoxic T-cells[70], fostering a robust antitumor immune response. In contrast, MSS tumors have an immunosuppressive microenvironment with regulatory T-cells (Tregs) and other immunosuppressive cell types that hinder effector T cell activity[71,72]. Eventually, the prognostic value of the immunoscore was initially established in individuals with colon cancer, showing its ability to assess prognosis based on factors such as the density, type, and localization of infiltrating immune cells[73].

ICIs are drugs that blocks certain key proteins on the surface of immune cells, particularly T-cells, and cancer cells, and release the brakes on the immune response. The development of ICIs has been a breakthrough in the field of cancer immunotherapy[36,69,74-76]. These proteins, known as immune checkpoints, play crucial roles in regulating the immune response. By blocking these checkpoints, ICIs enhance the ability of the immune system to recognize and attack cancer cells, thereby boosting the body's natural anti-cancer response[38]. ICIs have become a cornerstone of cancer therapy, with a wide range of approved agents available for multiple malignancies, leading to increased utilization in various treatment settings, including (neo)adjuvant and maintenance therapy. Thus, ICIs are accessible to nearly half of metastatic cancer patients in economically developed countries[77-79].

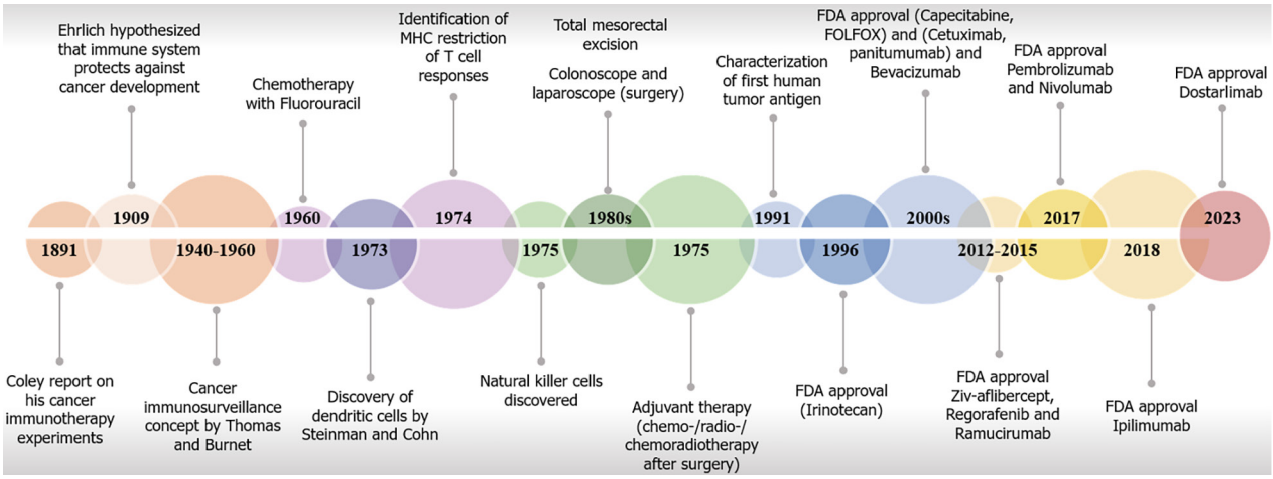


**Figure 1 Timeline with key milestones in immuno-oncology research and United States Food and Drug Administration–approved immune checkpoint inhibitors in colorectal cancer.**

## UNDERSTANDING THE MECHANISM OF IMMUNE CHECKPOINT INHIBITORS IN CRC

A solid understanding of the molecular drivers of CRC and identification of biomarkers of treatment response are essential for improving immunotherapy outcomes in patients with this disease[28,73,80-85]. A key element is the high TMB caused by genetic or sporadic mutations in MMR genes (such as MLH1, MSH2, MSH6, and PMS2), resulting in a deficiency of MMR proteins. This leads to an accumulation of genetic mutations in microsatellites[15,41], as observed in MSI-H or dMMR CRC tumors, compared to MSI-low (MSI-L) or pMMR. Consequently, enhanced immunogenicity is observed in such CRC cases, characterized by a higher count of neoantigens and substantial immune cell infiltration, including high numbers of tumor-infiltrating immune cells, such as CD8+ and CD4+ T-cells and macrophages[40,73,86-90]. Additionally, these tumors exhibit a microenvironment enriched with type I interferons, which distinguishes them from other CRC subtypes[87]. This immune-rich trait has been linked to improved rates of response to ICIs that block the PD-1/PD-L1 axis and T-cell activation[40,91]. In contrast, CRC tumors exhibiting pMMR along with MSS exhibit a low burden of mutations and low infiltration of CD4 and CD8 immune cells, resulting in evasion of the immune response.

ICIs enhance the recognition and elimination of cancer cells by activating the immune system, resulting in a more potent and sustained anticancer immune response. Understanding the mechanisms of synchronization with the disease pathophysiology is crucial for optimizing the therapeutic potential of ICIs and improving patient outcomes. The main mechanisms of action of ICIs include blockade of the PD-1/PD-L1 pathway and CTLA-4, which regulates T-cell activity and is often upregulated in tumors to evade the immune system (Figure 2)[38]. Indeed, PD-1 and CTLA-4 serve as negative regulators of T-cell activation and exert their biological effects at specific anatomical locations and at various points throughout the lifespan of T-cells[92]. The varied and late-onset autoimmune manifestations observed in Pdcd1−/− mice differ significantly from those in Ctla4-/- animals, highlighting that the PD1 axis governs T-cell biology in a distinct manner compared to CTLA4[92,93].

The PD-1/PD-L1 axis plays a role in autoimmunity by negatively regulating T-cell activation[94]. Functional loss of PD1 protein results in splenomegaly in mice models[94]. Additionally, mouse models lacking the PD-1 gene exhibit cardiac inflammation, leading to dilated cardiomyopathy and accelerated type 1 diabetes mellitus[95,96]. The PD1 axis is crucial for regulating differentiated effectors in T-cells[93,97,98]. Upon binding to PD-L1, PD1 exerts inhibitory intracellular signaling, leading to T-cell exhaustion, and eventually suppressing the immune response[99-101]. In addition to its role in regulating conventional T-cells, PD-L1 on antigen presenting cells (APCs) also plays a role in controlling Treg differentiation and immunosuppressive activity[102]. Tumor cells upregulate PD-L1 surface expression to take advantage of the PD-1/PD-L1 axis and escape immune response.

Anti-PD-1 antibodies such as pembrolizumab and nivolumab are ICIs used in cancer immunotherapy. Anti-PD-1 antibodies not only enhance the activity of cytotoxic T-cells but also affect the overall tumor microenvironment (TME) as well. These antibodies can alter the balance of immune cell populations by reducing the number of immunosuppressive cells such as Tregs and myeloid-derived suppressor cells[103]. This shift contributes to a more favorable immunological milieu for anti-tumor responses[104]. Anti-PD-1 antibodies promote increased production of pro-inflammatory cytokines, such as IFN-γ. These cytokines play key roles in amplifying the anti-tumor immune response by activating other immune cells and enhancing the recognition and elimination of cancer cells. In contrast, anti-PD-L1 antibodies target PD-L1 ligands on cancer cells. By blocking the interaction between PD-L1 and PD-1 on T-cells, these antibodies disrupt a key immune evasion mechanism employed by cancer cells. Anti-PD-1/PD-L1 antibodies help overcome adaptive immune resistance by enabling T-cells to recognize and target cancer cells more effectively. This leads to continuous adaptation of the immune response against evolving tumor cells. Immunotherapeutic responses are often associated with the expression of specific immunological biomarkers[105-107]. For anti-PD-1/PD-L1 therapy, the expression of PD-L1 on tumor cells is a commonly used biomarker. Tumors with high PD-L1 expression may have a higher likelihood of responding to anti-PD-1 antibodies. The presence of TILs is also considered a positive prognostic indicator of immuno-

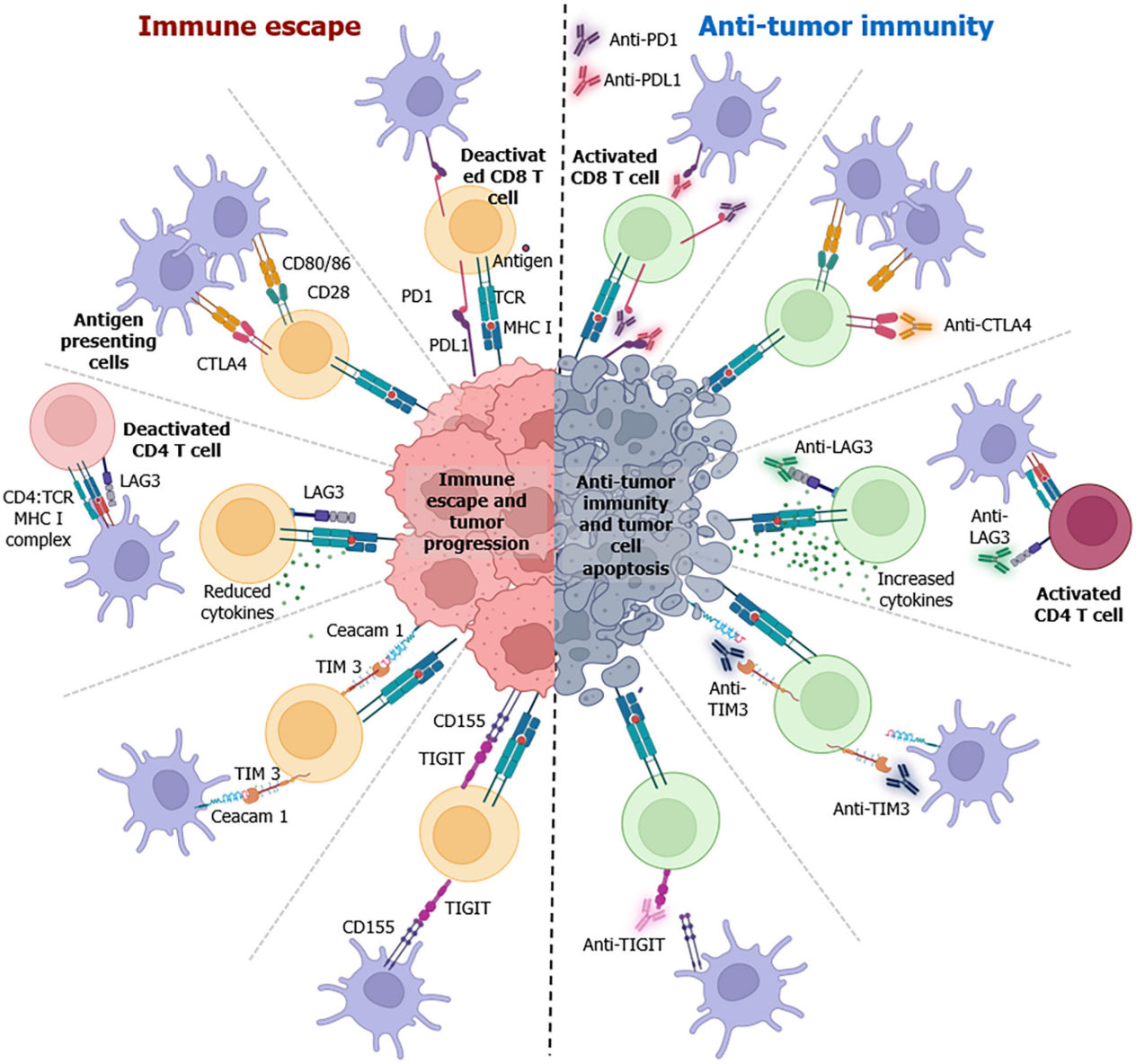


**Figure 2 This figure presents a schematic diagram of the intricate interplay between immune checkpoints, immune cells, and malignant cells.** It also elucidates the underlying molecular mechanisms employed in immune-checkpoint blockade. In the tumor microenvironment, the interaction of immune checkpoints leads to immune suppression and facilitates tumor progression (depicted on the left side of the diagram). Conversely, the administration of immune checkpoint inhibitions reverses the immune escape mechanism, fostering increased anti-tumor immunity and triggering tumor apoptosis (depicted on the right side of the diagram). FDA: Food and Drug Administration.

therapy response.

CTLA-4, a vital immune checkpoint, exhibits low basal levels in conventional T-cell. However, its expression is significantly induced after antigen activation. Activated T-cells expressing CTLA4 impede the interaction between B7-1 and B7-2 molecules on APCs and CD28, and thereby induce anergy and reduce T-cell activation[108-110]. TCR signaling studies affirm CTLA4's role in inhibiting T-cell activation and proliferation[111-113]. Ctla4-knockout mice were found to develop T-cell mediated autoimmune disease, which is mitigated by treatment with the CTLA4: Fc fusion protein (CTLA4Ig)[114-116]. Notably, CD4+ CD25+ Tregs, which are known for their immunosuppressive function, constitutively express CTLA4 and are necessary for the release of anti-inflammatory cytokines from Tregs[117,118]. These findings confirm that CTLA4 is a T-cell activation inhibitor with potential as a therapeutic agent against cancer[119]. Pre-clinical studies using anti-CTLA-4 antibodies aimed to prevent inhibitory signals, allowing for a more effective CD28 interaction with B7[120]. However, the results were found to depend on tissue specificity and tumor size[119,121]. Additionally, blocking CTLA4 enhances T-cell responses to tumor-associated neoantigens, and a high neoantigen burden predicts a positive response to anti-CTLA4 therapy.

Blocking CTLA-4 with anti-CTLA-4 antibodies promotes a sustained and enhanced T-cell activation. CD28 is a co-stimulator of T-cell activation that benefits from the increased availability of anti-CTLA-4 antibodies and facilitates enhanced binding to B7. This then amplifies co-stimulatory signals, promoting T-cell proliferation and function[120]. These antibodies also modify the TME by decreasing immunosuppressive cells, such as Tregs, creating a more favorable setting for anti-tumor immune responses[122,123]. Additionally, anti-CTLA-4 antibodies induce antibody-dependent cell-

mediated cytotoxicity, with immune cells, particularly natural killer cells, recognizing and eliminating target cells marked with therapeutic antibodies[124]. Often used in combination with other ICIs, such as anti-PD-1 or anti-PD-L1 antibodies, this approach targets multiple checkpoints simultaneously, enhancing the overall anti-tumor immune response. Anti-CTLA-4 therapy induces systemic immune activation, affecting not only the tumor site but also distant metastases, contributing to the potential for durable responses and efficacy.

ICIs can also enhance antigen presentation by dendritic cells (DCs), and thus facilitate the priming of T-cells to initiate a robust and targeted immune response against cancer[125,126]. Activated DCs present tumor antigens to T-cells effectively to promote T-cell activation and proliferation. Adaptive immune resistance involves a dynamic interplay between the immune system and cancer cells. Successful ICIs therapy is associated with memory T-cell generation. Memory T-cells contribute to long-term immune memory by enabling the immune system to respond rapidly to cancer cell recurrence. Understanding these immunological nuances provides insights into how ICIs contribute to unleashing and potentiating the ability of the immune system to recognize and eliminate cancer cells. Despite its success in MSI-H CRC, the clinical efficacy of immunotherapy in MSS CRC remains very limited[28]. Recent studies have found that characteristics such as high levels of TILs and expression of immune checkpoint molecules such as PD-L1 may help identify patients with MSS CRC who are more likely to benefit from ICI treatment.

## CLINICAL TRIALS INVOLVING CHECKPOINT INHIBITORS IN CRC

The emergence of checkpoint inhibitors has brought a remarkable shift in our approach to treatment of cancers, including CRC. Notably, these inhibitors have exhibited encouraging treatment outcomes in specific subsets of patients, such as those with MSI-H or dMMR tumors, which are characterized by augmented levels of TILs and heightened susceptibility to immune checkpoint blockade. This section focuses on noteworthy clinical studies on application, efficacy, and potential benefits of ICIs in CRC. In 2014, the Food and Drug Administration (FDA) approved Pembrolizumab, a PD-1 immune checkpoint inhibitor for melanoma treatment[127]. Tumor cells evade the immune system through the PD-1 pathway where PDL1 and PDL2 Ligands on tumor cells binds to the PD-1 receptors on T cells to inactivate T cells. Pembrolizumab binds to these PD-1 receptor and blocks their interaction with PDL1 and PDL2, thereby restoring the immune response [128]. Subsequently, in 2020, the FDA approved this drug for patients with unresectable or metastatic MSI-H or dMMR CRC, based on results from key clinical trials. Phase 2 open-label, multicenter trial (NCT01876511) was conducted to evaluate the safety, efficacy, and tolerability of pembrolizumab in MSI-H-positive patients[129]. Trials have shown no dose-limiting toxicities associated with pembrolizumab, with a promising disease control rate of 80% in patients with MSI-positive CRC, suggesting the potential of Pembrolizumab in CRC treatment. Subsequently, another trial (NCT02460198) postulated the efficacy of pembrolizumab in patients with unresectable tumors who underwent standard chemotherapy[130,131]. The results showed a promising overall progression response rate of 32 to 34 months in both cohorts. This study demonstrated the potential of pembrolizumab as an effective treatment option for patients with dMMR and MSI-H mCRC.

Chemotherapy has been used over the years for the treatment of patients with CRC to shrink tumor volume[132]. In 2015, a phase 3 clinical trial (NCT02563002) was conducted to test pembrolizumab as a first-line treatment in comparison with standard chemotherapy treatment in mCRC patients with MSI-H or dMMR tumors[133,134]. The results showed a significant improvement in the progression-free survival (PFS) rate of 16.5 months in comparison to the standard chemotherapy group at 8.2 months. The trial demonstrated pembrolizumab monotherapy to be superior to standard chemotherapy in terms of PFS and overall response rate (ORR) for patients with MSI-H or dMMR mCRC as a standard care option. The potential efficacy of pembrolizumab in these patients was demonstrated by increased production of neoantigens resulting from an elevated mutational burden. This, in turn, leads to a heightened recognition of tumor cells by cytotoxic T cells, which are primed by blocking the interaction between PD-1 and PD-L1. These results thus led to a paradigm shift in the treatment of this patient population.

The evolution of pembrolizumab has led to the development of more PD-1-targeting drugs whose efficacy and safety profiles were assessed. Nivolumab, another PD-1 monoclonal antibody, was approved in 2017 for use in mCRC treatment [28]. Both drugs exhibited similar modes of action in blocking PD1 and inducing increased CTLs cytotoxicity. However, these two antibodies also exhibited significant structural differences in their binding to PD-1[135]. The epitope region of pembrolizumab displayed a considerably larger overlap with the PD-L1 binding site compared to that of nivolumab. Notably, the binding sites of pembrolizumab and nivolumab on PD-1 showed almost no convergence[135]. A study published in 2017 compared the effectiveness of drugs with comparable effectiveness, which may potentially be interchangeable. The effectiveness of nivolumab has been studied in NCT02060188 MSI-H or dMMR mCRC patients with or without the CTLA-4 inhibitor drug Ipilimumab[136-138]. The treatment showed promising results, with a disease control rate of 80% with nivolumab alone. Combination treatment with a CTLA-4 inhibitor was effective in 51 of the 74 patients who achieved disease control for a minimum of 12 wk. However, further studies are still needed to determine the optimal treatment duration for pembrolizumab and to identify predictive biomarkers of response to immunotherapy in this population. Overall, Nivolumab plus ipilimumab combination therapy is a promising treatment with a better disease control rate and objective response.

Atezolizumab, a monoclonal antibody targeting PD-L1, was approved by the FDA in 2016 for the treatment of non-small cell lung cancer tumors[139]. The mechanism of action of Atezolizumab differs from those of pembrolizumab and nivolumab. Instead of binding to PD-1, atezolizumab binds to PD-L1 on tumor cells, and thereby provides a mode of action similar to that of the PD-1 antibody. A clinical trial (NCT02788279) was conducted to evaluate the efficacy of this drug alone and in combination with a MEK inhibitor, cobimetinib, compared to regorafenib (a multi-kinase inhibitor)

[140]. This combination is used as a therapeutic alternative to a MEK inhibitor that increases T-cell proliferation and CD8+ T cell infiltration, and PD-1 treatment to upregulate the PD-1/PD-L1 interaction, thereby downregulating the immunosuppressive TME. The results of the trial showed an improved PFS rate 1.91 of 2 months after cobimetinib treatment. In conclusion, combination of atezolizumab and cobimetinib improved PFS and ORR compared to regorafenib as a second-line treatment for patients with mCRC, yet did not significantly improve OS. These results underscore the potential of combination therapy and suggest improved investigations of combined immunotherapy drugs as promising targeted treatment approaches for mCRC. Combination therapy with MEK inhibition and PD-L1 blockade led to impressive long-term survival rates. MEK inhibitors act during the post-naïve stage of T-cell differentiation. MEK inhibition counteracts the expression of Nur77, which is associated with exhaustive T cell death induced by antigen-specific $CD8^+$ T cells, thereby rescuing T cell exhaustion[16].

Another PD-1 monoclonal antibody, dostarlimab, was approved in 2021 and tested in clinical trials (NCT04165772) in patients with MSI-H or dMMR CRC[141]. A high-resolution structure revealed that Dostarlimab binds to the flexible loops of PD-1, including the BC, C'D, and FG loops, in contrast to the binding modes of Pembrolizumab or Nivolumab [94]. The initial findings of the trials were published for 12 patients. Accordingly, all the patients (100%; 95%CI: 74-100) showed a complete clinical response[142]. The response was confirmed using magnetic resonance imaging, which showed no evidence of tumors. At that time, none of the patients had received chemoradiotherapy or undergone surgery. No cases of progression or recurrence were observed during follow-up (6–25 months). The study listed no grade 3 or higher AEs during the trial period. This study thus demonstrated the high sensitivity of dMMR locally advanced rectal cancer to single-agent PD-1 blockade. Despite these promising results, a longer follow-up period and a larger sample size are still needed to assess the duration of the response.

Following the improved success of ICIs in CRC patients with MSI-H or dMMR tumors, researchers have investigated their efficacy in MSS or pMMR CRC patients as well. These data suggested that more than 85% of CRC patients with MSS tumors show resistance to ICIs therapy. A clinical trial (NCT01876511) illustrating the potential of pembrolizumab in MSI-H or dMMR CRC patients has shown no measurable responses in any of the 18 patients with pMMR CRC, as defined by the RECIST criteria[129]. To overcome these limitations, combination treatments have been used to improve the drug responses. A clinical trial was conducted using PD-L1 with a multi kinase inhibitor in patients with MSS or pMMR CRC. Regorafenib targets stromal and oncogenic receptor tyrosine kinases, and shows anti-angiogenic activity due to its dual-target VEGFR2-TIE2 tyrosine kinase inhibition[143]. The NCT04126733 trial conducted between 2019 and 2022 included 94 CRC patients with MSS or pMMR[144]. The results showed an ORR of 7% and overall survival (OS) rate of 11.9%. The relatively reduced success observed in patients with MSS or pMMR CRC indicates the need for further advancement of drug efficacy to provide better outcomes in MSS tumors.

In 2016, a combination trial (NCT01988896) was performed in CRC patients with BRAF/KRAS mutations using cobimetinib and atezolizumab to study OS and PFS in MSS or pMMR CRCs patients[145]. These mutations are rarely identified but are more frequently found in patients with MSS CRC. BRAF and KRAS mutations are mutually exclusive, and BRAF-mt induces aberrant and inappropriate activation of the MAPK/ERK pathway, making it a good candidate for combination therapy with ICIs and kinases or MEK inhibitors[146]. As expected, the combination provided relatively better outcomes in patients with BRAF/KRAS mutations, with an OS rate of 43%. These findings provide compelling evidence that MAPK pathway blockade therapy combined with ICIs is promising for improving treatment efficacy in mCRC patients with MSS/pMMR BRAF mutations.

Recent developments have improved targeted immunotherapies using engineered ICIs to increase the success rate in patients with MSS or pMMR CRC[147]. A phase 1 clinical trial was conducted to categorize the adverse effects and dose-limiting toxicity of botensilimab, an Fc-engineered anti-CTLA-4 monoclonal antibody, in patients with MSS CRC. This Fc engineering promotes intratumoral Treg depletion and reduces complement fixation. This modification provides optimized T-cell priming, activation, and memory formation by strengthening antigen-presenting cell/T-cell co-engagement. The trial showed an ORR of 22% (95%CI: 12-35) and a disease control rate of 73% (95%CI: 42-75) for patients with non-hepatic disease in refractory CRC. The trial showed the efficacy of the anti-tumor activity in MSS CRC patients with active liver metastatic disease, and a phase 2 trial (NCT05608044) for MSS CRC has begun to study its potential in controlling tumor progression.

The success of ICIs in patients with MSI-H CRC has been hindered by their reduced potential in MSS CRC. The results from clinical trials in patients with MSS CRC undergoing immune checkpoint blockade immunotherapy suggest the need for the development of new pre-clinical mouse models to replicate the microenvironment of human CRC, and potentiate new targeted therapies to improve patient survival.

## LIMITATIONS OF USING IMMUNE CHECKPOINT INHIBITORS FOR IMMUNOTHERAPY

Although ICI therapy holds benefits, patients also often experience autoimmune-like effects known as immune-related adverse events (irAEs). This is likely the result of generalized, non-antigen-specific activation of the immune system following a checkpoint blockade. Inhibition of a naturally occurring central immune checkpoint releases potent immune effector mechanisms that may not adhere to the usual boundaries of immune tolerance towards self-tissues[148]. Human loss-of-function mutations in CTLA4 and its regulatory partner, LPS Responsive Beige-Like Anchor Protein, mimic the immune-related side effects of anti-CTLA4 therapy[149,150]. irAEs have been reported in 15%–90% of patients[57], with severe events requiring intervention being observed in 30% and 15% of patients treated with CTLA4 and PD1 axis inhibitors, respectively[151]. This immune checkpoint inhibitor leads to toxicity in naïve T cells and accumulation of memory T cells in peripheral organs[152,153]. Compared with PD-1, CTLA4 therapy possess with severe autoimmune

complications, as seen in pre-clinical and clinical trials[154].

Colitis is a frequent complication observed in ICIs therapy[155]. Anti-CTLA4 treatment resulted in a potentially higher colitis rate, ranging from 5.7% to 22% of patients, compared to 0.7% to 1.6% with anti–PD-1 agents[156]. The development of ICI-mediated colitis and diarrhea (IMC) may involve cytotoxic CD8+ T cells. Recent analysis of single-cell RNA sequences from patients with IMC revealed an expansion of tissue-resident memory CD8+ T cells into inflammatory populations within the colon tissue, suggesting that the activation or alteration of CD8+ T cell populations could be a potential mechanism for colitis induced by ICIs[157]. Therefore, with the potential use of immune checkpoint blockade, the current research should aim to identify potential predictive markers for organ-specific toxicities caused by immunotherapy.

## IMMUNE PROFILING OF MSI AND MSS CRC INFLUENCE ICIS SUCCESS

Patients comprising MSI-H or dMMR tumors have a significantly high overall mutation burden, with approximately 12 mutations per million DNA bases. In contrast, pMMR/MSI-L tumors have a relatively reduced tumor burden, with fewer than eight mutations per million DNA bases[158]. This phenomenon is primarily attributed to somatic defects in the function of MMR genes, with the most prevalent mechanism being hypermethylation of the MLH1 promoter, which serves as a prognostic marker. In MSI, frameshift mutations in protein-coding sequences can create diverse peptides that serve as potential neoepitopes, which are recognized as foreign by the immune system. Mutant peptides form complexes with major histocompatibility complex class I molecules and act as foreign neoantigens that initiate immune cell priming and infiltration. Within the TME, tumor-associated macrophages play a crucial role in influencing tumor growth and progression. Recent study has demonstrated a frameshift mutation in the TGFβRII producing an immunogenic peptide called p538[159]. This peptide is present in over 90% of tumors with dMMR, indicating its broad relevance in the field. These tumors exhibit robust infiltration by immune cells, particularly CD8+ TILs, Th1 CD4+ TILs, and macrophages[73]. Furthermore, the microenvironment of these tumors is notably enriched in type I interferons, which distinguishes them from other CRCs types.

Approximately 15% of all CRCs exhibit MSI-H or dMMR characteristics[160]. Patients with MSI-H or dMMR tumors before ICIs therapy continue to have a poor prognosis. Cancers show significantly upregulated expression of PD1, PDL1, and CTLA4, rendering them potentially susceptible to ICIs[87]. In contrast, MSS or pMMR tumors lacking neoantigens are characterized by reduced T cell infiltration and elevated levels of immunosuppressive ligands. These characteristics offer insights into the disagreement between MSI-H or dMMR and MSS or pMMR CRCs in ICIs responses and could potentially serve as prognostic biomarkers for patient selection. As shown in previously described clinical trials, immunotherapy as a neoadjuvant approach has not shown any clinical benefit in patients with MSS or pMMR CRC, including individuals with mCRC.

Contrastingly, the MSS CRC, majorly referred to as an "immune cold" cancer type, are predisposed by various molecular factors contributing to the underlying resistance to immunotherapy[161]. MSS-CRC is characterized by larger chromosomal aberrations that mark the phenotype of MSS-CRC, resulting in a lower tumor mutation burden and reduced neoantigen configuration. This framework partially elucidates the disparate clinical responses to ICIs observed in these CRC subgroups. The MSS CRC TME hosts more tumor-associated macrophages, which have been associated with poor prognosis in most studies. Notably, a pioneer study identified that increased β-catenin activation (a downstream effect of APC mutation) resulted in reduced infiltration of CD8+ and CD103+ DCs, orchestrating an immune suppressive environment *via* T-cell exclusion[16]. The APC protein is mutated in more than 70% and 20% of MSS and MSI-H CRCs, respectively, driving the distinct oncogenesis mechanisms and subsequent “immune hot” and “immune cold” TME[162]. These differences are reflected in clinical trials with ICIs, where MSS tumors have very low response rates compared to MSI tumors (Table 1).

## IMPLICATION OF PRE-CLINICAL MOUSE MODELS OF CRC IN DRUG DEVELOPMENT AND IMMUNOTHERAPY

Mouse- and cell-based models have been used for decades to investigate the molecular origins of CRC, and more recently, to identify drug and immune responses in specific CRC types (Figure 1). These efforts have yielded tremendous improvement in our basic understanding of the disease and TME. However, despite recent approvals, the majority of patients continue to have limited immunotherapy options. A recurring challenge highlighted in the literature is the absence of a mouse model that precisely replicates the progression of human CRC from adenoma and adenocarcinoma to metastasis, including changes in the microenvironment. Initial mouse models lacked significant penetrance of the metastatic phenotype, often forming tumors in the small intestine rather than in the colon, unless induced by laparoscopy [163,164]. In 2013, the National Institute of Health formally concluded the Mouse Models in Human Cancer Consortium, leading researchers to explore alternative models, such as patient-derived xenografts and patient-derived organoids, to study the disease. The significance of the TME in metastasis remains a focus of current research, and the potential of checkpoint inhibitors and other immunological and inhibitor therapies are being explored. However, an ideal model still does not exist, highlighting the importance of an immunocompetent autochthonous model. Notably, single-cell RNA sequencing of mouse tumors to understand the mechanisms underlying immune-modulating therapies could help draw more impactful conclusions[165]. Despite these limitations, the diversity of the methods employed by researchers with

**Table 1 List of clinical trials with immune check point inhibition therapy**

| Trial identified (number of patients) | Treatment groups | Patients enrolled | Primary and secondary outcomes |
|---|---|---|---|
| Checkpoint inhibitor: Pembrolizumab | | | |
| NCT01876511 (*n* = 113) | Pembrolizumab | Cohort A: MSI positive (pMMR) CRC | Cohort A: ORR: 54.0% (95%CI: 37.0–69.0); PFS: 70% (95%CI: 57–86); OS: NA (95%CI: 151.86-NA) |
| | | Cohort B: MSI negative (dMMR) CRC | Cohort B: ORR: 0% (95%CI: 0.0–14.0); PFS: 16% (95%CI: 6–41); OS: 36.71 (95%CI: 21.29-69.43) |
| NCT02460198 (*n* = 124) | Pembrolizumab | mCRC with dMMR or MSI–H status Cohort A: Participants must have received prior treatment with standard therapies | Cohort A: ORR: 32.8 (95%CI: 21.3 to 46.0); PFS: 2.3 (95%CI: 2.1–8.1); OS: 31.4 (95%CI: 21.4–58) |
| | | Cohort B: Participants must have undergone at least one line of systemic standard of care therapy | Cohort B: ORR: 34.9 (95%CI: 23.3–48.0); PFS: 4.1 (95%CI: 2.1–18.9); OS: 47 (19.2–NA) |
| NCT02563002 (*n* = 307) | Arm A: Pembrolizumab | mCRC with high MSI–H or dMMR | Arm A: ORR: 45.1% (95%CI: 37.1–53.3); PFS: 16.5 (95%CI: 5.4–38.1); OS: NA (95%CI: 49.2–NA) |
| | Arm B: mFOLFOX6/FOLFIRI/Bevacizumab/Cetuximab/Pembrolizumab | | Arm B: ORR: 33.1% (95%CI: 25.8–41.1); PFS: 8.2 (95%CI: 6.1–10.2); OS: 27.6 (95%CI: 27.6–NA) |
| Checkpoint inhibitor: Nivolumab + Regorafenib | | | |
| NCT04126733 (*n* = 94) | Regorafenib and Nivolumab | Patients with pMMR or MSS CRC | ORR: 7% (95%CI: 2.4–15.9); PFS: 1.8 (95%CI: 1.8–2.4); OS: 11.9 (95%CI: 7.0 to NA) |
| Checkpoint inhibitor: Nivolumab + Ipilimumab | | | |
| NCT02060188 (*n* = 119) | Arm A: Nivolumab | MSI–H or dMMR mCRC | Arm A: No results posted |
| | Arm B: Nivolumab + Ipilimumab | | Arm B: ORR: 55% (95%CI: 45.2%–63.8%); PFS: 71% (95%CI: 61.4 to 78.7); OS: 85% (95%CI: 77.0 to 90.2) |
| | Arm C: Cobimetinib + Nivolumab + Ipilimumab | | Arm C: No results posted |
| | Arm D: Nivolumab + Daratumumab | | Arm D: No results posted |
| | Arm E: Nivolumab + BMS–986016 | | Arm E: No results posted |
| Checkpoint inhibitor: Atezolizumab | | | |
| NCT02788279 (*n* = 363) | Arm A: Atezolizumab | Patients with mCRC (MSI or MSS status unknown) | Arm A: PFS: 1.94 (95%CI: 1.91 to 2.10); OS: 7.10 (95%CI: 6.05–10.05) |
| | Arm B: Cobimetinib + Atezolizumab | | Arm B: PFS: 1.91 (95%CI: 1.87 to 1.97); OS: 8.87 (95%CI: 7.00–10.61) |
| | Arm C: Regorafenib | | Arm C: PFS: 2 (95%CI: 1.87–3.61); OS: 8.51 (95%CI: 6.41–10.71) |
| NCT01988896 (*n* = 84) | Atezolizumab + Cobimetinib | Patients having BRAF/KRAS mutation in mCRC | ORR: 8% (7/84) (6 patients: MSS, 1 patient: MSI) |
| NCT01633970 (*n* = 10) | Arm A: Atezolizumab + Bevacizumab | No results posted | No results posted |
| | Arm B: Atezolizumab + Bevacizumab + FOLFOX | | |
| | Arm C: Atezolizumab + Carboplatin + Paclitaxel | | |

| | | | |
|---|---|---|---|
| | Arm D: Atezolizumab + Carboplatin + Pemetrexed | | |
| | Arm E: Atezolizumab + Carboplatin + Nab–paclitaxel | | |
| | Arm F: Atezolizumab + Nab–paclitaxel | | |
| Checkpoint inhibitor: Durvalumab + Tremelimumab | | | |
| NCT03122509 (*n* = 25) | Arm A: Durvalumab + Tremelimumab + Radiotherapy | Metastatic Colorectal Cancer (MSI or MSS status unknown) | Arm A: ORR: 8%; Stable response: 12%; Progressive disease: 76% |
| | Arm B: Durvalumab + Tremelimumab + Ablation | | Arm B: No participants enrolled |
| Checkpoint inhibitor: Dostarlimab | | | |
| NCT04165772 (*n* = 200) | Arm A: Dostarlimab | Patients with dMMR rectal adenocarcinoma | Arm A: Complete response: 12 patients (100%; 95%CI: 74–100) |
| | Arm B: Dostarlimab + Capecitabine or 5–FU + IMRT | | Arm B: No participants enrolled |

CRC: Colorectal cancer; mCRC: Metastatic colorectal cancer; MSI: Microsatellite-instability; MSI-H: Microsatellite instability-high; pMMR: Proficient mismatch repair; dMMR: Deficient mismatch repair; MSS: Microsatellite-stable; 5–FU: 5-fluorouracil IMRT: Intensity modulated radiotherapy.

mice, the adaptability of the system, and the deductive formation of CRC images from diverse models remain impressive.

Various transplantation techniques have been introduced over the years to replicate complex TME in mouse models. The subcutaneous injection model has been widely used[166], yet it has limitations, such as creating an ectopic environment and lacking accuracy in mimicking the metastatic spread of cancer[167]. The orthotopic transplantation model has emerged as a promising alternative to address these issues. This involves a precise injection into the intestinal region, such as the cecal wall, colon, or rectum. Among these, the orthotopic CRC model using cecal wall injections has been widely adopted. However, it is essential to recognize that even this model has constraints as it does not faithfully replicate the anatomical location where tumors typically occur in humans and exhibits a microenvironment distinct from the rest of the colon[168].

Genetically engineered mouse models (GEMMs) are based on genetic engineering techniques, particularly the Cre recombinase lox-P system, to simulate tumorigenesis by modifying the structure of the genome[169]. GEMMs, including those incorporating mutations in genes, such as *APC*, *KRAS*, *p53*, and *MSH2*, provide valuable insights into the molecular mechanisms underlying CRC and play a pivotal role in the study of CRC development and therapeutic strategies. APC mutations activate Wnt/b-catenin, causing increased b-catenin levels and tumor development[170,171], restricting tumor growth within the intestines, and mimicking human CRC. In addition, compared with previous single-mutation GEMMs for CRC, transgenic mice established *via* combined APC/KRAS mutations have been shown to represent CRC initiation, progression, and metastasis more accurately into nearby tissues[163,172]. Advantages include specificity in mirroring human CRC growth, representation of TMEs, and the ability to visualize the CRC through colonoscopy. Immunotherapeutic studies using transgenic mice have revealed promising avenues for targeted treatments. However, limitations such as extended metastatic development time and limited colon-specific models warrant further refinement for comprehensive CRC research[173]. As a result, transgenic mice are often ineffective in representing the later stages of tumor development owing to highly variable metastasis[166]. Additionally, there are few current transgenic mouse models that lead to specific CRC development in the colon, as the majority of pre-existing models lead to CRC development within the small intestine or other nearby tissues, contributing to the development of familial cancers rather than specific GEMM mutation-derived CRC[174].

The observation that tumors in mice have a narrower phenotype than human tumors suggests that the mice themselves need to be subtyped before drawing comparisons with human subtypes. This recognition is crucial, especially considering that murine backgrounds are often congenic (with the same genetic makeup) and artificially altered for experimental purposes. In summary, the critical importance of selecting appropriate preclinical models in CRC research requires better understanding. Mouse models are indispensable tools for discovering effective therapeutic interventions. The evaluation of various transplantation methods, with particular emphasis on the orthotopic CRC model *via* cecal wall injection, provides a nuanced understanding of their utility while acknowledging the inherent limitations associated with this approach. This recognition is vital for refining experimental design and interpretation to better translate findings from preclinical studies to human clinical scenarios.

## FUTURE DIRECTIONS

Overall, immunotherapy, particularly with the use of ICIs like pembrolizumab and nivolumab has demonstrated significant clinical benefit in MSI-H CRC, while its efficacy in MSS CRC remains limited[91,130,133,138]. However, even in MSI-H tumors, the upregulation of immune checkpoint proteins, the presence of other immunosuppressive mechanisms within the TME, and the heterogeneity of MSI-H CRC tumors within the primary tumor and across

metastatic sites can contribute to varied responses to immunotherapy. Understanding these factors and further research on the mechanisms of immune resistance in patients with MSI-H CRC are essential to improve the outcomes of immunotherapy in this patient population.

In the field of ICI therapy for CRC, various research avenues to enhance treatment efficacy and broaden its scope of application are being actively pursued. Therefore, there is a need to identify response biomarkers and devise novel treatment approaches to address these challenges. One area of focus is the investigation of combination therapies in which ICIs are used in conjunction with chemotherapy, targeted therapies, and other immunotherapies. Similarly, combining immunotherapy with targeted therapy directed against specific signaling pathways, such as the MAPK pathway, may also improve treatment outcomes[136-138,140,175]. Here, the goal is to enhance the response rates and improve patient survival by leveraging the synergistic effects of different treatment modalities. Another important research avenue is the discovery of reliable biomarkers that can accurately predict patient response to ICIs. Although PD-L1 expression is currently used as a biomarker for some cancer types[176,177], its predictive value for CRC is limited[129,178]. For example, tumors with elevated PD-L1 expression may be more responsive to anti-PD-1/PD-L1 therapy, whereas tumors with low PD-L1 expression may require combination therapy to achieve a response. Similarly, tumors with specific genetic mutations such as BRAF V600E may require targeted therapy in addition to immunotherapy to achieve a response. Mutations in genes such as *JAK1*, *JAK2*, and *B2M* may contribute to treatment resistance[179-181]. Truncating mutations in B2M affect antigen presentation, and can lead to pembrolizumab resistance. Evidence indicates that a high somatic mutational load and neoantigen density are associated with an improved response to immune checkpoint blockade in various cancers. This is attributed to the increased presence of mutation-associated neoantigens, which contribute to greater T cell diversity[182]. Moreover, there are also ongoing research efforts on the development of novel immunotherapeutic agents such as bispecific antibodies, chimeric antigen receptor (CAR) T-cells, and vaccines, which may provide new treatment options for CRC patients[183-188]. Efforts are underway to identify additional biomarkers that assist patient selection and treatment decisions. These targeted therapies, when combined with combination therapies, have shown considerable potential in enhancing treatment efficacy and overcoming drug resistance. By simultaneously targeting different pathways implicated in CRC progression, these approaches aim to maximize therapeutic benefits while minimizing adverse effects. Future research should focus on identifying optimal drug combinations, elucidating synergistic interactions, and refining treatment regimens to improve patient response.

A recent study revealed that immune cells form multicellular hubs in CRC samples that are spatially organized and functionally distinct from the surrounding immune cells[85]. The findings indicated that these immune hubs are composed of different cell types, including T-cells, B-cells, and myeloid cells, and are enriched in specific functional pathways related to the immune response and cell-cell communication. Researchers have also observed that the distribution and composition of immune hubs vary between patients and may be influenced by factors such as tumor stage and treatment history. Furthermore, the findings also demonstrated that the presence of immune hubs was associated with better clinical outcomes in CRC patients, suggesting a crucial role in the immune response to cancer. The authors proposed that targeting immune hubs could be a promising strategy for enhancing the efficacy of immunotherapy in CRC[85].

Additionally, there is a growing interest in exploring the use of ICIs in the early stages of CRC, such as adjuvant therapy following surgery. Early detection and intervention are pivotal for improving CRC outcomes. Emerging technologies, such as liquid biopsies and advanced imaging modalities, hold promise for the detection of CRC at earlier stages when treatment options are more effective[189-191]. Additionally, minimally invasive surgical techniques and organ-preserving approaches offer less invasive alternatives for managing early stage CRC, reducing morbidity, and improving the quality of life of patients[192].

Current research focuses on understanding the intricate mechanisms underlying drug resistance, including genetic mutations, TME interactions, and adaptive signaling pathways. Strategies for overcoming resistance include developing combination therapies that target multiple pathways, repurposing existing drugs, and developing novel agents to evade resistance mechanisms. Precision medicine approaches such as tumor molecular profiling and real-time monitoring facilitate the early detection of resistance mechanisms, allowing prompt adjustments to treatment strategies[193,194]. Furthermore, biomarker research in CRC is rapidly evolving with the aim of identifying molecular signatures crucial for diagnosis, prognosis, and treatment decisions. Biomarkers, such as mutations in genes such as KRAS and BRAF, not only influence tumor behavior, but also affect responses to targeted therapies, notably anti-EGFR antibodies[24]. Additionally, MSI status serves not only as a guide for immunotherapy but also as a valuable prognostic indicator as well. Liquid biopsies offer a noninvasive method to analyze circulating tumor DNA and to monitor disease progression and treatment responses[189]. Epigenetic alterations, such as DNA methylation patterns and microRNA expression profiles, are promising diagnostic and prognostic markers[24,195]. Traditional biomarkers, such as carcinoembryonic antigen, provide insights into tumor burden and treatment response, while gene expression signatures, such as Oncotype DX and ColoPrint, offer predictive value for treatment outcomes and recurrence risk assessment[196]. Integrating these diverse biomarkers into clinical practice can help personalize treatment strategies, optimize patient management, and ultimately enhance the survival outcomes for CRC patients. However, drug resistance remains a significant challenge, compromising the efficacy of chemotherapy, targeted therapies, and immunotherapy[132]. CRC cells develop resistance to chemotherapeutic drugs such as fluoropyrimidines and oxaliplatin through mechanisms such as altered drug metabolism and enhanced drug efflux[197,198]. Similarly, targeted therapies may encounter resistance due to secondary mutations or activation of alternative signaling pathways. Understanding and overcoming these mechanisms are crucial for advancing CRC treatment and improving patient prognosis. Finally, new targets and agents beyond the PD-1/PD-L1 pathway are being investigated. Promising preclinical data to have led to clinical trials of molecules targeting additional T cell checkpoint inhibitors, such as TIM3, LAG3, and TIGIT, in various advanced malignancies, including CRC (Figure 2)[94,199-202]. In addition to immune checkpoint blockade, molecules that enhance T-cell differentiation, survival, and prolif-

eration are being investigated as standalone treatments or in combination with checkpoint inhibitors. These molecules, including CD27, OX40, 4-1BB, and others, act as antibody agonists for the costimulatory group within the TNF receptor superfamily[203-209].

Personalized medicine has revolutionized CRC treatment by tailoring interventions to individual patient characteristics, including genetic and molecular factors. Over the years, personalized medicine has gained traction and treatment approaches have been tailored based on individual patient characteristics. This involves integrating tumor genetic profiling, immune profiling, and other personalized medicine strategies to identify the most effective treatment options for each patient[194,210,211]. Comprehensive genomic profiling and clinical data integration enable the identification of actionable targets and personalized treatment regimens. Artificial intelligence enhances data interpretation and improves the accuracy of treatment response prediction. Liquid biopsies provide a noninvasive method for monitoring disease progression and identifying therapeutic targets. Personalized medicine integrates liquid biopsy-based monitoring into treatment management, allowing for real-time therapy adjustments. This approach promises to optimize treatment strategies and improve the clinical outcomes for CRC patients with CRC. Advancements in biomarker research coupled with efforts to overcome drug resistance and implement personalized medicine offer a multifaceted approach to CRC management that holds great promise for enhancing patient care and outcomes in the future.

Moreover, additional novel strategies for CRC treatment such as mRNA vaccines, TILs therapy, CAR-T therapy, oncolytic virus therapy, bispecific T-cell engagers, and combination strategies aim to improve treatment outcomes by specifically considering metastatic location and TME regulation. Novel agents and therapeutic strategies are being developed to expand the range of options available for immune modulation of this disease. Accordingly, in addition to the development of new biomarkers and therapeutic strategies, there is also a need for better pre-clinical mouse models that can potentially or closely replicate the human CRC microenvironment, thus providing a better opportunity to unmask novel approaches for treatment. As the field of immunotherapy evolves, these directions hold great promise for advancing immune checkpoint inhibitor therapy and other immunotherapeutic approaches for CRC, ultimately resulting in improved patient outcomes.

## CONCLUSION

Immunotherapy has significantly reshaped the CRC treatment landscape, particularly for patients with MSI-H or dMMR tumors. Key accomplishments include the FDA approval of PD-1 inhibitors, such as pembrolizumab and nivolumab, for these patient subsets. Pembrolizumab has demonstrated promising outcomes both as a monotherapy and in combination with chemotherapy, surpassing standard treatments in terms of PFS and ORR[133,134]. Furthermore, combination therapies have shown promise, such as the use of nivolumab with ipilimumab (a CTLA-4 inhibitor), which has demonstrated improved disease control rates[136-138]. Additionally, atezolizumab in combination with Cobimetinib has shown enhanced PFS rates in second-line treatments, although further studies are needed to establish its effects on OS [145]. A recent study established Dostarlimab as a drug with 100% effectiveness against MSI-H or dMMR CRC tumors. Despite the preliminary success of immuno-oncology, challenges persist for CRC treatment, particularly those pertaining to the extension of immunotherapeutic benefits to MSS or pMMR tumors, which commonly exhibit resistance to ICIs. In this regard, irAEs associated with ICIs should be managed effectively, which requires identification of predictive biomarkers and the development of mitigation strategies. Combination therapies, as exemplified by the synergistic effects observed with nivolumab and ipilimumab, require further investigation to optimize their performance and to identify their underlying mechanisms. Exploring novel therapeutic targets beyond immune checkpoint blockade, including targeted therapies and engineered immunotherapies, holds promise for overcoming resistance mechanisms. Addressing these challenges requires interdisciplinary collaboration, ongoing preclinical and clinical research, and rigorous validation through well-controlled trials. By overcoming these obstacles, advancements in CRC treatment can be realized, leading to improved clinical outcomes and enhanced quality of life in affected patients. In conclusion, immunotherapy has revolutionized CRC treatment, resulting in improved outcomes and survival rates in MSI-H or dMMR patients. However, challenges persist in extending these benefits to patients with MSS or pMMR and in the management of irAEs. Future research should focus on optimizing combination therapies, identifying predictive biomarkers, and mitigating treatment-related toxicities to realize the full potential of immunotherapy in CRC management.

## FOOTNOTES

**Co-first authors:** Samantha Sharma and Naresh Singh.

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**Country/Territory of origin:** United States

**ORCID number:** Samantha Sharma 0000-0001-9599-4455; Naresh Singh 0000-0003-4051-4084.

**Corresponding Author's Membership in Professional Societies:** American Association for Cancer Research, 1215631.

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*REVIEW*

# Hepatolithiasis: Epidemiology, presentation, classification and management of a complex disease

Rodrigo V. Motta, Francesca Saffioti, Vasileios K Mavroeidis

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**Rodrigo V. Motta,** Translational Gastroenterology and Liver Unit, Nuffield Department of Medicine, University of Oxford, Oxford OX3 9DU, United Kingdom

**Francesca Saffioti,** Department of Gastroenterology and Hepatology, Oxford University Hospitals NHS Foundation Trust, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

**Francesca Saffioti,** UCL Institute for Liver and Digestive Health and Sheila Sherlock Liver Unit, Royal Free Hospital and University College London, London NW3 2QG, United Kingdom

**Vasileios K Mavroeidis,** Department of HPB Surgery, Bristol Royal Infirmary, University Hospitals Bristol and Weston NHS Foundation Trust, Bristol BS2 8HW, United Kingdom

**Vasileios K Mavroeidis,** Department of Transplant Surgery, North Bristol NHS Trust, Southmead Hospital, Bristol BS10 5NB, United Kingdom

**Vasileios K Mavroeidis,** Department of Gastrointestinal Surgery, North Bristol NHS Trust, Southmead Hospital, Bristol BS10 5NB, United Kingdom

**Corresponding author:** Vasileios K Mavroeidis, MD, MSc, FRCS, FACS, FICS, FSSO, MFSTEd, MICR, Academic Research, Surgeon, Department of HPB Surgery, Bristol Royal Infirmary, University Hospitals Bristol and Weston NHS Foundation Trust, Upper Maudlin St, Bristol BS2 8HW, United Kingdom. vasileios.mavroeidis@nhs.net

## Abstract

The term hepatolithiasis describes the presence of biliary stones within the intrahepatic bile ducts, above the hilar confluence of the hepatic ducts. The disease is more prevalent in Asia, mainly owing to socioeconomic and dietary factors, as well as the prevalence of biliary parasites. In the last century, owing to migration, its global incidence has increased. The main pathophysiological mechanisms involve cholangitis, bile infection and biliary strictures, creating a self-sustaining cycle that perpetuates the disease, frequently characterised by recurrent episodes of bacterial infection referred to as syndrome of "recurrent pyogenic cholangitis". Furthermore, long-standing hepatolithiasis is a known risk factor for development of intrahepatic cholangiocarcinoma. Various classifications have aimed at providing useful insight of clinically relevant aspects and guidance for treatment. The management of symptomatic patients and those with complications can be complex, and relies upon a multidisciplinary team of hepatologists, endoscopists, interventional radiologists and hepatobiliary surgeons, with the

main goal being to offer relief from the clinical presentations and prevent the development of more serious complications. This comprehensive review provides insight on various aspects of hepatolithiasis, with a focus on epidemiology, new evidence on pathophysiology, most important clinical aspects, different classification systems and contemporary management.

**Key Words:** Cholelithiasis; Intrahepatic stones; Cholangiocarcinoma; Biliary parasites; Recurrent pyogenic cholangitis; Oriental cholangiohepatitis; Hepatectomy; Cholangioscopy; Liver transplant; Paediatric



**Core Tip:** Hepatolithiasis is a chronic disease, mostly prevalent in Asia, commonly characterised by recurrent episodes of cholangitis and relevant clinicopathological syndromes, while it constitutes a risk factor for development of intrahepatic cholangiocarcinoma. Its management in complex cases can be challenging and relies upon multidisciplinary input from hepatologists, endoscopists, interventional radiologists and hepatobiliary surgeons. This comprehensive review provides insight on various aspects of hepatolithiasis, with a main focus on the epidemiology, new evidence on pathophysiology, most important clinical aspects, different classification systems and contemporary management.

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## INTRODUCTION

Cholelithiasis is the general term describing the presence of gallstones at any point along the biliary tree. Cholecystolithiasis is the precise term that describes the presence of gallstones within the gallbladder, even though in clinical practice, very often the terms cholelithiasis and gallstones are used for the same purpose, owing to the gallbladder being the predominant anatomical location of gallstones. The term choledocholithiasis refers to the presence of gallstones within the extrahepatic bile ducts, while hepatolithiasis describes the presence of gallstones within the intrahepatic bile ducts[1,2] (Figure 1).

More specifically, hepatolithiasis is characterised by the development of pigmented stones, mostly composed of calcium bilirubinate and less of cholesterol, in the intrahepatic bile ducts before their confluence into the common hepatic duct, irrespective of the coexistence of gallstones in the common bile duct (CBD) and/or gallbladder. The pathogenesis involves several mechanisms, including bile infection, cholestasis, hyperplasia of the biliary epithelium and bile duct strictures, all of which increase the risk of cholangiocarcinoma (CCA) in these patients[3,4]. In the case of primary hepatolithiasis, in the absence of extrahepatic biliary obstruction intrahepatic stones often cause chronic inflammation, mural fibrosis, and proliferation of peribiliary glands[5]. Frequently, hepatolithiasis is characterised by recurrent episodes of bacterial infections of the biliary system also referred to as "recurrent pyogenic cholangitis"[6]. The management of the disease currently relies on interventional procedures, to relieve cholestasis and extract impacted stones, as well as liver resection in cases of severe biliary strictures and hepatic parenchymal atrophy. This comprehensive review provides insight on various aspects of hepatolithiasis, with a focus on epidemiology, new evidence on the pathophysiology of the disease, most important clinical aspects, different classifications and contemporary management.

## EPIDEMIOLOGY

The disease was described in Hong Kong in 1930[7], and is endemic to East and Southeast Asia, where its reported prevalence has been as high as 30%[8]. For these reasons, it has also been described as "oriental cholangiohepatitis", "oriental cholangitis", or "Hong Kong Disease"[9,10].

The incidence of hepatolithiasis varies greatly even within Asian countries, and Taiwan has the highest relative prevalence historically. Parasitic infections may play an important role in the development of hepatolithiasis[11]. In 1961, Fung[12] identified that among 262 patients treated with hepatolithiasis in Hong Kong, 30% also had liver fluke. More recently, economic development, improvements in the overall hygiene and introduction of a western diet richer in proteins and saturated fats, have led to a reduction in the incidence of the disease[13]. Nevertheless, a Taiwanese retrospective nationwide study in the early 90's confirmed a high prevalence of hepatolithiasis among the 17182 patients included, with figures around 20%[14]. On the other hand, data available from Japan, showed a decrease in the incidence of hepatolithiasis in the past century, from 4.1% between 1970 and 1977 to 1.7% in 1995[10].

Glenn and Moody[15] reported in 1961 that patients of Asian descent living in the United States had a similar incidence of hepatolithiasis to the general American population, suggesting that the pathogenesis of the disease is mainly related to

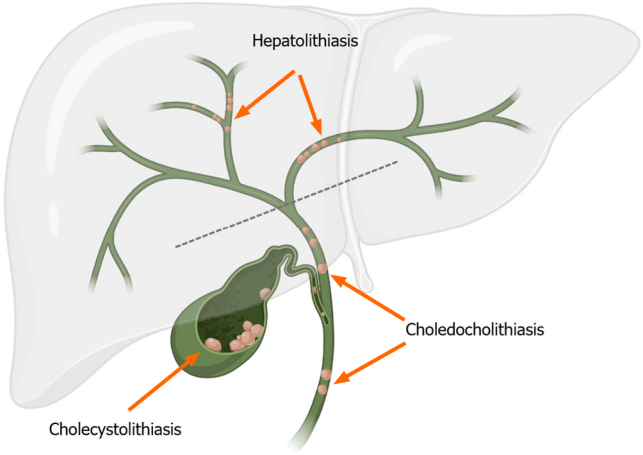


**Figure 1 Topographic variations of cholelithiasis.** The dashed line at the hilar ductal confluence delineates the level of differentiation between hepatolithiasis and choledocholithiasis.

environmental, rather than genetic and ethnic factors. This also reinforces the theory that improved living standards and a change in the dietary habits play a major role in the decreased incidence.

Most systematic analyses of incidence and prevalence of hepatolithiasis come from Asia, although the disease is also prevalent among Latin Americans, and has been associated with lower socioeconomic status and rural environments[13,16]. A Brazilian group reported an incidence of 2.1% among patients referred to a tertiary centre for treatment of biliary stones[17].

In Western countries, the disease is rare and usually presents as secondary hepatolithiasis, i.e., associated with underlying conditions causing strictures on the bile ducts and/or stasis of the bile in the biliary tree, such as primary sclerosing cholangitis, malignancy, post-surgical complications, or choledochal cysts[16]. According to historical data, the prevalence of hepatolithiasis in the West is < 2%, although in the last century, migration has increased its global incidence overall[16]. A Swedish observational study on autopsies reported that among patients with gallstones, 1.9% had stones in the hepatic duct and 0.6% had intrahepatic stones[18]. The disease appears to be more frequent in women, and an age > 50 years has been reported as a risk factor[19].

## PATHOPHYSIOLOGY AND RISK FACTORS

The pathophysiology of hepatolithiasis is complex and still uncertain. Cholestasis, biliary infection, abnormal bile duct anatomy, changes in bile metabolism, malnutrition and diet are factors that possibly drive and perpetuate the disease[3,20,21]. Patients with anatomical and functional changes in the biliary system have a higher risk of hepatolithiasis. Among these, congenital and/or acquired intrahepatic bile duct deformities (*e.g.*, Caroli's disease, primary sclerosing cholangitis, anastomotic stricture) are major risk factors. The presence of strictures creates non-linear trajectories of bile flow, thus facilitating the agglutination of crystals in the bile ducts and development of stones[22]. The left hepatic duct and the right posteroinferior bile duct are areas prone to the development of cholestasis when their angulation hinders the bile flow[23]. Moreover, a dysfunctional sphincter of Oddi has a negative effect on the homeostasis of the biliary tree. Once it has lost its one-way valve function, duodenal fluid can reflux into the ampulla of Vater and reach the CBD. This fluid will alter the biliary flora and cause inflammation on the bile ducts due to differences in the pH and the presence of pancreatic enzymes[22].

Studies have shown a possible association between bacterial translocation, bile infection and biliary stones[24,25]. Commensal microorganisms normally found in bile ducts are *E. coli, S. typhimurium, B. cereus and L. monocytogenes*[26]. Continuous bile flow, the sphincter of Oddi and the presence of immune cells in the biliary tree are natural mechanisms to keep these bacteria from triggering an infection. However, Xiao *et al*[27] observed that 40 Chinese patients with hepatolithiasis had a different bile flora when compared to the control group, with a larger population of *Proteus* and *Streptomyces*. Furthermore, microbiological studies observed that the diversity of bacteria in the biliary tract of patients with hepatolithiasis is smaller than the diversity in controls, thus suggesting an imbalance in the flora[27]. Biliary dysbiosis, specifically the increased presence of anaerobic bacteria, is related to changes in the bile homeostasis[28] leading to a higher risk of intrahepatic stones formation. Moreover, larger populations of lipopolysaccharide-producing bacteria trigger signalling pathways that lead to an increased production of mucin 5AC by cholangiocytes[29]. The higher concentration of mucin, in turn, promotes cholestasis and crystallisation of cholesterol[30,31]. In addition, bacteria produce beta-glucuronidase, or induce its production, leading to deposition of calcium bilirubinate and stone formation [22,32,33]. Finally, these microorganisms also produce phospholipases, responsible for breaking phosphatidylcholine into free fatty acids, consequently accelerating the deposition of fatty acid calcium and promoting further production of mucin by the cholangiocytes[27,34]. All of these mechanisms together create a self-sustaining cycle and promote stone formation.

Parasite infection, mainly by *Ascaris*, *Clonorchis sinensis*, *Opisthorchis viverrini*, and *Schistosoma*, is also associated with hepatolithiasis. These organisms usually live in the intestines but are known for invading the biliary tree. Once parasites pass the sphincter of Oddi and reach the bile ducts, they incite various mechanisms to fight helminths, such as increased intestinal epithelial cell turnover[35], and a switch from production of Mucin 2 to Mucin 5AC in the gut[36]. Moreover, parasites might cause mechanical obstruction of the bile drainage and promote further cholestasis. Finally, calcium bilirubinate crystals attach to helminth eggs, which represent an ideal substrate for formation of biliary stones[37].

Metabolomic analyses observed that patients with hepatolithiasis have different serum levels of lipids and lipid-like molecules from healthy controls[38]. This reinforces previous findings that a dysregulated lipid metabolism is directly involved with the development of intrahepatic biliary stones[29,39,40]. Furthermore, mutations in ABCB4 and ABCB11, genes associated with bile production and mucin secretion, also have a role in the development of intrahepatic stones[22,41]. Cystic fibrosis (CF) is a common genetic disease caused by approximately 2000 mutations in the *CFTR* gene, the most prevalent of which is ΔF508. Notably, patients with the disease have a high incidence of cholelithiasis of 15–30% as opposed to an age-matched population incidence of 5%. CF is one of the very few diseases, which cause hepatolithiasis in children. Patients with large duct disease in particular, develop strictures, cholangiectasis, hepatolithiasis and recurrent infections[42].

The amount of protein and fat in the diet directly influences secretion of bile. East Asian populations tend to adopt low protein and low-fat dietary patterns, thus negatively affecting bile excretion and favouring cholestasis[22]. Low-protein diets also cause reduction of beta-glucuronidase inhibitors in the bile. Once cholestasis, biliary infection, anatomical abnormalities, and bile metabolism changes are in place, they create a vicious cycle of biliary injury. Recurrent damage to the bile ducts leads to activation of myofibroblasts, periductal lamellar fibrosis and hyperplastic epithelium, thus creating biliary strictures[43]. The hepatic parenchyma adjacent to impacted stones is usually marked with different degrees of atrophy and fibrosis according to the level of inflammation in that area. If biliary obstructions are left untreated, the chronic cholestasis can lead to biliary cirrhosis although it is not a common finding[43].

## CLINICAL FEATURES

### *Clinical presentation*

The signs and symptoms of hepatolithiasis are associated with the degree of cholestasis and presence of biliary infection. Patients will often complain about abdominal discomfort, nausea and vomiting, but may also present with cholangitis and the classic Charcot's triad of abdominal pain, fever and jaundice[44]. Complicated cases can present with Reynold's pentad (*e.g.*, abdominal pain, fever, jaundice, confusion and hypotension), suggesting organ dysfunction, which is a marker of poor prognosis[20]. On the other hand, some patients can be oligo- or asymptomatic and receive a diagnosis of hepatolithiasis as an incidental finding during investigations for different conditions or due to non-specific abdominal symptoms[20].

Changes in the physical examination will depend on the underlying activity of cholangitis and biliary obstruction. Usual findings are epigastric discomfort on palpation and hepatomegaly[44].

### *Complications*

**Complex hepatolithiasis:** One of the most common complications is the development of complex hepatolithiasis, when the disease affects both lobes and/or intrahepatic stones are associated with hilar stenosis/distortion[45]. If left untreated, biliary stenosis will lead to upstream chronic cholestasis, predispose the sedimentation of bile salts and formation of stones[22]. The treatment of hepatolithiasis, therefore, must involve the resolution of any bile duct strictures and promote normal bile flow.

**Persistent cholangitis:** Laxity of the sphincter of Oddi and biliary strictures increase the risk of infections in the biliary tree. The presence of bacteria also favours precipitation of bile salts and formation of stones[22], leading to a self-sustaining cycle of cholangitis, hyperplasia of the biliary epithelium, subsequent strictures, and further stone formation [43]. The biliary flora dysbiosis associated with a substrate that favours proliferation of pathogenic bacteria facilitates the recurrence of cholangitis, thus explaining why this disease is also called "recurrent pyogenic cholangitis".

**Biliary cirrhosis:** Up to 14.1% of patients develop secondary biliary cirrhosis[3,46,47] and its complications, including portal hypertension and liver failure. Cirrhosis occurs as a consequence of the chronic inflammatory injury to the bile ducts, where the recurrent biliary sepsis may lead to periductal inflammation, fibrosis and portal thrombophlebitis[46]. The treatment includes management of the complications (ascites, portal hypertensive bleedings, hepatic encephalopathy, *etc.*) and, in the more advanced cases, orthotopic liver transplantation (OLT)[48,49].

**CCA:** Hepatolithiasis is a risk factor for intrahepatic CCA, which occurs in up to 21.2% of cases[3,50-52]. Intrahepatic CCA is an aggressive cancer with poor prognosis, particularly as complete surgical resection is only possible in less than a third of patients[53]. Patients with hepatolithiasis that develop intrahepatic CCA have higher levels of c-erbB2, epidermal growth factor, COX-2 and nuclear factor-kB, which are biomarkers of chronic inflammation[54-56]. Furthermore, tumour suppressor genes, namely p16 and DPC4/Smand4, are commonly inactivated in these patients, thus reinforcing the role of chronic inflammation in carcinogenesis, as seen in patients with primary sclerosing cholangitis.

The Japanese Hepatolithiasis Research Group followed 401 patients with hepatolithiasis for 18 years. This cohort had a 2.0% cumulative incidence of CCA at 5 years, 4.0% at 10 years and 6.2% at 15 years of follow-up[51]. Among these patients, independent risk factors for intrahepatic CCA were age ≥ 63 years (HR 3.334, 95%CI: 1.316-8.499), residual

stones after treatment (HR 2.445, 95%CI: 1.047-5.711) and the occurrence/formation of biliary stricture during follow-up, (HR 4.350, 95%CI: 1.821-10.391). The incidence of CCA was higher in patients with three risk factors than in those with one or two risk factors[51]. Moreover, a Chinese retrospective study of 981 patients who underwent hepatectomy for hepatolithiasis, identified 55 cases with intrahepatic CCA over a median follow-up of 65 months. The authors reported that residual stones (HR 2.101, 95%CI: 1.150–3.839), formation of hepaticojejunostomy (HR 1.837, 95%CI: 1.077-3.133), and uniformity between extension of liver resection and stone-affected segments (i.e., whether all of the affected segments or less were resected) (HR 2.442, 95%CI: 1.205-4.948) are also independent factors for intrahepatic CCA[57]. In addition, a case-control study from China reported that smoking (OR = 1.931, 95%CI: 1.000-3.731), family history of cancer (OR= 5.175, 95%CI: 1.216-22.022) and symptoms for more than 10 years (OR= 2.348, 95%CI: 1.394-3.952) were independent risk factors for development of CCA in patients with hepatolithiasis[52].

### *Paediatric population*

Data on aspects of hepatolithiasis in the paediatric population are extremely limited, particularly as primary hepatolithiasis is very rare in children. Yue[58] in 1974 was the first to publish a case series of 6 children with recurrent pyogenic cholangitis, treated between 1952 and 1972 at a single centre in Hong Kong, China, "with the main object of introducing this disease entity". Four were males and 2 were females, with ages ranging from 7-14 years. In all cases soft pigment stones were found while in one case non-viable Ascaris lumbricoides was also detected. All patients received emergency surgical exploration, and 2 patients required re-operation. All patients were alive at a follow-up of 3-19 years. The author highlighted that the treatment of this entity in children is based on experience with adult patients[58].

Saing *et al*[59] in 1988 published their experience with 10 children treated with "recurrent pyogenic cholangitis", at their hospital in Hong Kong, China, between 1973 and 1984. The presentation was uniformly acute with cholangitis, including features of septicaemia. Initial management included intravenous fluids and electrolytes, antibiotics against aerobic and anaerobic intestinal bacteria, and nasogastric suction, in all cases. While 4 of the 10 children responded well to conservative treatment, 3 of them subsequently required elective transduodenal sphincteroplasty. The remaining 6 patients required emergency surgery owing to poor response to conservative management after 24-48 h, and/or progression to septic shock. One patient required re-exploration. One death occurred following emergency sphincteroplasty owing to septicaemia, pulmonary complications, and pericardial effusion. At exploration, pigment stones and/or mud were found in the bile ducts in all cases. On two occasions Ascaris lumbricoides was found in the CBD. During follow-up over 3-12 years, the children remained well and continued to grow satisfactorily. Three patients required re-admissions for mild abdominal pain, which resolved with conservative measures[59].

As mentioned earlier, hepatolithiasis in childhood may also develop in patients with CF, particularly those with large duct disease[42].

A single-centre Spanish case-series from 1992 reported 7 paediatric patients with intrahepatic stones between 1981-1989, and retrospectively evaluated their radiological features. There were 6 female and 1 male patient in this cohort with a mean age of 3.6 years. Underlying causes were present, including CF in one case, immunodeficiency syndrome in a further case, 4 cases of extrahepatic biliary atresia treated with portoenterostomies, and a case following partial liver transplantation[60]. Of note, a recent retrospective study of 301 paediatric patients who underwent living donor liver transplantation over a 20-year period at a single centre in Japan, reported development of biliary strictures in 18%. During endoscopic treatment 23 patients (7%) were found to have developed hepatolithiasis and were managed with endoscopic techniques. However, a high recurrence rate of 30% was observed[61].

Recently, a Chinese single-centre retrospective cohort study of 106 children with a discharge diagnosis of "lithiasis", hospitalized between 2010-2021, that were diagnosed with primary hepatolithiasis, reported male preponderance, mean age at diagnosis of 9.3 ± 3.6 years, and a preferential involvement of the right liver lobe (75.5%). The estimated incidence of primary hepatolithiasis in children was approximately 1.7 per 10000 hospitalized patients. All patients were asymptomatic and had hepatolithiasis discovered randomly on abdominal ultrasound during their treatment of other diseases; however, 6 patients had undergone previous choledochal cyst excision. More than 25% of patients diagnosed with primary hepatolithiasis had elevated γ-GGT. The authors noted that most of the underlying diseases that resulted in hospitalisation required treatment with antibiotics or hormones, suggesting that the formation of intrahepatic stones in children might be related to the use of these drugs; however, the pathogenesis of hepatolithiasis in this age group remains to be clarified. All patients were managed conservatively under observation. During follow-up of approximately 8 years, none of the patients developed severe clinical symptoms or complications and no patient required surgical intervention in childhood[62].

## INVESTIGATIONS

Laboratory tests are not very informative for hepatolithiasis apart from showing the levels of systemic inflammation, cholestasis and biliary/hepatic injury.

Imaging studies, however, are paramount to identify the location of stones, complications and determine the best treatment. Ultrasound and magnetic resonance cholangiopancreatography (MRCP) are the preferred methods because both can detect non-calcified stones. While ultrasound is usually the first investigation, MRCP offers a more accurate evaluation of the biliary tree and is better at detecting biliary strictures and dilations[10,63,64]. Non-calcified stones cannot be identified on computerised tomography, but the method is useful to locate abscesses and biliary dilations[10].

Biomarkers are not particularly useful for the diagnosis of hepatolithiasis. Recently, Wang *et al*[38] identified four metabolites, namely 18-β-Glycyrrhetinic acid, FMH, Rifampicin and PC (4:0/16:2), that are over-expressed in hepato-

lithiasis and had a good efficacy to discriminate patients with hepatolithiasis from healthy controls. However, the study only included 30 Chinese patients, thus these biomarkers need validation in larger and more heterogeneous populations before they are used in clinical practice.

## CLASSIFICATION

Throughout the years, many classifications have been developed to distinguish between cases of hepatolithiasis. One of the most used is Dong's classification, that proposes a treatment approach depending on the location of intrahepatic stones, hepatic atrophy, biliary changes, presence of extrahepatic stones and sphincter of Oddi function[65]. Both Nakayama's classification and the Tsunoda classification focus on describing the disease based on location of stones, presence and location of biliary stricture and dilation[66,67]. Finally, the recently proposed LHO system[68] divides patients according to their capacity to tolerate an anatomical hepatectomy, the presence of hilar strictures and changes at the sphincter of Oddi. A summary of the criteria in each of these classifications are presented in Table 1.

### *Dong's classification*

Feng *et al*[65] reported results from a cohort of 2000 patients being treated for hepatolithiasis in 2012. They proposed a classification method based on location of the stones, presence of hepatic atrophy, biliary stricture, cirrhosis, and the function of the sphincter of Oddi.

Hepatolithiasis type I is characterised by localised stones and is subdivided into Ia, when stones are in one lobe, and Ib, when stones are in both lobes. Type II is defined by diffuse disease and has three subtypes: type IIa has no hepatic atrophy or bile duct stenosis, type IIb has segmental atrophy and/or biliary strictures, and type IIc has biliary cirrhosis and portal hypertension. Type E is used to characterise disease with extrahepatic stones and is divided into Ea, when the sphincter of Oddi has normal function, Eb when there is relaxation of the sphincter, and Ec when there is stenosis of the sphincter of Oddi.

### *Nakayama's classification*

In 1982, Nakayama reported his classification criteria for hepatolithiasis[66]. This is solely based on a thorough and objective description of the type of stones, location of stones, presence of biliary stenosis, its severity and site, and presence of biliary dilation, its severity and location. This classification also includes whether the gallbladder contains stones, whether there is dysfunction of sphincter of Oddi and whether the patient has undergone previous operations.

The severity of biliary stenosis is graded as S0 when there is none, S1 when the related bile duct's diameter is more than 2 mm (mild stricture), and S2 when the bile duct's diameter is less than 2 mm (marked stricture). Biliary dilation is graded using D0 for absence, D1 for dilation less than 20 mm, and D2 for dilation more than 20 mm.

Although being informative and profoundly descriptive, it has to be noted that this method may be difficult to interpret to those that are not often exposed to patients with hepatolithiasis. Furthermore, the classification does not suggest the preferred method of treatment which could guide surgeons with less expertise in hepatolithiasis.

### *Tsunoda classification*

Tsunoda *et al*[67], in 1985, published a method to classify patients with hepatolithiasis. These criteria divided patients based on primary and secondary hepatolithiasis, the location of stones, the presence of biliary strictures and/or dilations. According to each classification, they recommended which operation would lend the best surgical outcomes.

Patients with type I have small stones and no dilation of bile ducts, while patients with type II have diffuse dilation of intrahepatic ducts with or without an obstructive lesion on the CBD. Both types are associated with concomitant choledocholithiasis. The Tsunoda classification suggests that these patients might develop hepatolithiasis due to an extrahepatic factor and called both types as secondary intrahepatic stones. Type III is characterised by unilateral cystic lesions with or without strictures, and type IV is represented by diffuse disease occupying both lobes.

### *LHO Classification System*

The most recent classification method was published by Wang *et al*[68] in 2023. They named it LHO Classification System, where each letter indicates one of the three key points assessed to formulate an effective surgical plan and standardize the treatment of patients with hepatolithiasis. The first letter, L, assesses the distribution of the stones in the segmental bile ducts, the presence of hepatic atrophy and patient's tolerance to anatomical hepatectomy. H refers to the presence or absence of stones at the hilum or hilar strictures, and O indicates whether or not there is dysfunction of the sphincter of Oddi.

With regards to the subtypes of type L, L0 refers to the absence of obvious stones in the segmental bile ducts or obvious atrophy of the hepatic parenchyma, along with normal liver function. In subtype L1, there are stones in the segmental bile ducts and/or parenchymal atrophy and the patient can tolerate hepatectomy. Type L2 is also defined as complex hepatolithiasis, as it indicates diffuse disease and atrophy, without feasibility for anatomical hepatectomy of all the affected segments within the safe limits of resectability according to the criteria of the University of Zurich[69].

Type H refers to strictures and obstructive stones at the liver hilum. These need to be removed to prevent chronic cholestasis and recurrent cholangitis/stone formation. H0 indicates the absence of hilar stones or strictures. Subtype H1 means that there are stones but no strictures around the hepatic hilum, while H2, instead, refers to the presence of both stones and hilar strictures and includes cases that are usually more difficult to manage.

**Table 1 Main classifications of hepatolithiasis and criteria accounted for**

| Classification | Criteria | Advises on treatment |
|---|---|---|
| Dong's classification[65] | Location of stones | Yes |
| | Presence of hepatic atrophy | |
| | Presence of biliary strictures | |
| | Cirrhosis | |
| | Function of the sphincter of Oddi | |
| Nakayama's classification[66] | Type of stones | No, entirely descriptive |
| | Location of stones | |
| | Presence of biliary stenosis, severity, and location | |
| | Presence of biliary dilation, severity, and location | |
| | Presence of stones inside the gallbladder | |
| | Function of the sphincter of Oddi | |
| | Previous operations | |
| Tsunoda classification[67] | Primary or secondary lithiasis | Yes |
| | Location of stones | |
| | Presence of biliary strictures | |
| | Presence of biliary dilation | |
| LHO classification system[68] | Tolerance to anatomical hepatectomy | Yes |
| | Distribution of stones | |
| | Presence of parenchymal atrophy | |
| | Presence of hilar stricture | |
| | Function of the sphincter of Oddi | |

Type O varies according to the functionality of the sphincter of Oddi, as its insufficiency may lead to duodenal fluid reflux into the biliary tree and recurrent cholangitis. Subtype O0 indicates normally functioning sphincter, in subtype O1 there is relaxation of the sphincter and subtype O2 refers to patients with a stricture of the sphincter of Oddi.

## MANAGEMENT

Treatment of hepatolithiasis follows a stepwise approach and depends on symptoms and extension of hepatic/biliary involvement. Asymptomatic patients usually get diagnosed during investigations for other conditions. If there are no signs of cholangitis, biliary stricture and hepatic atrophy, as well as no history of biliary reconstruction, these patients can undergo active monitoring with regular follow-up using non-invasive imaging (*e.g.*, ultrasound and magnetic resonance imaging)[70].

Symptomatic patients and those with complications should be managed by a multidisciplinary team involving hepatologists, hepatobiliary endoscopists, interventional radiologists and hepatobiliary surgeons[64]. If there is evidence of biliary strictures or history of biliary reconstruction, these patients require a more aggressive management, including invasive methods to remove stones and dilate stenotic ducts. Cases where hepatic atrophy is present should ideally undergo hepatectomy[71]. The most important goals during treatment of hepatolithiasis are removal of stones, resolution of strictures and prevention of cholangitis to hinder progression of the disease and development of CCA[72].

A schematic summary of the main treatments of hepatolithiasis according to clinical presentation is shown in Figure 2.

### *Pharmacotherapy*

Pharmacological treatment for hepatolithiasis lacks large controlled prospective studies, hence it is not recommended. Ursodeoxycholic acid (UDCA) decreases the secretion of cholesterol in the bile and inhibits intestinal reabsorption of cholesterol, while it facilitates bile flow *via* stimulation of hepatobiliary secretory function[73]. It has been used to prevent gallstone formation in cases of cholelithiasis/choledocholithiasis, as well as in cases of hepatolithiasis due to Caroli's syndrome[74]. In primary hepatolithiasis, however, stones are commonly composed of calcium bilirubinate rather than cholesterol[75]. Even though it addresses part of the mechanism of stone formation, UDCA does not tackle the mechanisms leading to the deposition of bile crystals and formation of calcium bilirubinate stones.

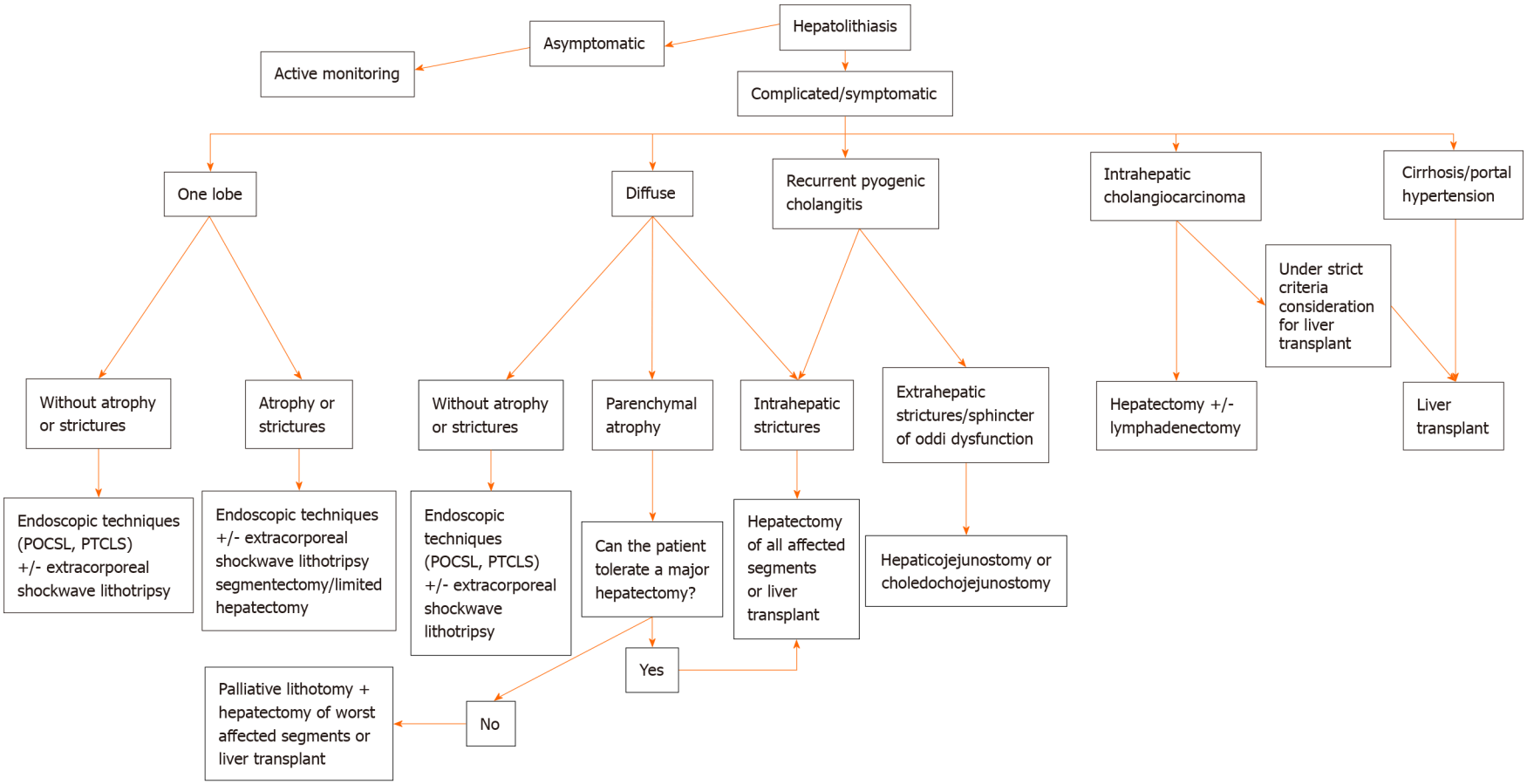


**Figure 2 Schematic summary of the main treatments of hepatolithiasis according to clinical presentation.** POCSL: Peroral cholangioscopy with lithotomy; PTCSL: Percutaneous transhepatic cholangioscopic lithotomy.

### *Interventional management*

Interventional treatment for hepatolithiasis involves various techniques, such as peroral cholangioscopy (POCS)[76], percutaneous transhepatic cholangioscopic lithotomy (PTCSL)[77], and percutaneous transhepatic balloon dilation[78].

POCS, associated or not with lithotomy has gained increased importance in the treatment of hepatolithiasis[79,80]. In a recent study evaluating the efficacy and safety of POCS (SpyGlass), technical success was achieved in 71.4% and clinical success in 57% of cases[81]. Similar results were previously reported in a larger retrospective study comparing the outcomes of hepatectomy ($n$ = 90), PTCSL ($n$ = 97) and POCS ($n$ = 49)[46]. Complete stone clearance was achieved in 83.3% of hepatectomies, 63.9% of PTCSL, and 57.1% of POCS patients. PTCSL has a higher success rate, achieving complete stone removal in up to 85% of the cases, but with a recurrence rate reaching more than 50%[46,82-85]. Moreover,

both POCS and PTCSL can also be used in association with extracorporeal shockwave lithotripsy to improve stone clearance rates to 90%[86,87]. It is important to highlight, however, that the presence of hepatic atrophy and/or biliary strictures hinders the success of these therapies, with complete stone removal rates of only 58%[88].

### Surgical management

Hepatectomy reduces the risk of stone recurrence and progression to CCA[21,89]. Hence, in patients with bilateral disease, hepatic atrophy and who can tolerate a more major operation, anatomical resection of all liver segments affected by the disease is the ideal treatment. The surgical access (open *vs* minimally invasive) will depend mainly on whether the patient can tolerate pneumoperitoneum, as well as the expertise of the surgeon. There is evidence supporting non-inferiority of a laparoscopic approach on mortality, and superiority on acute endpoints like length of hospital stay and blood loss[90-94].

In 2021, Kim *et al*[95] developed and validated a difficulty scoring system (DSS) for laparoscopic liver resection. This considers location of the stone, atrophy of the hepatic parenchyma, extent of liver resection, ductal stricture within 1 cm of the bifurcation and combined choledochoscopic examination for remnant intrahepatic ducts. The score ranges between 3 and 12 points, with highest scores indicating increased level of surgical difficulty. When patients were divided into three subgroups according to DSS (DSS 3-5, DSS 6-8, and DSS 9-12), hospital stay ($P = 0.05$), operation time ($P < 0.001$) and blood loss ($P = 0.004$) were significantly different between the three subgroups.

Complicated hepatolithiasis is commonly characterised by intrahepatic stones with hepatic atrophy and hilar stenosis/distortion[45] It is essential that a multidisciplinary team gets involved in the treatment. Often patients will present with persistent stones after hepatic resection and almost 70% will also present with extrahepatic stones[5,96]. Therefore, it is important that hepatobiliary endoscopists, interventional radiologists and hepatobiliary surgeons define the best line of action to achieve complete stone removal as well as resolution of biliary strictures and resection of atrophic segments. These cases can be managed with a combination of peroral, or percutaneous lithotomy associated with hepatectomy of the most affected segments. Jiang *et al*[97] used laser lithotripsy combined with or without hepatectomy, achieving a final stone clearance rate of 93.3%. In very complex cases, patients might need to be considered for liver transplantation. Up to 15.6% of patients will require bile duct exploration after hepatectomy, due to incomplete stone clearance[98,99] and as many as 38.5% can present with recurrent hepatolithiasis[100]. These patients are also at high risk of developing biliary cancer, with as many as 9.7% been diagnosed with concomitant CCA[101], and up to 12.2% developing subsequent CCA during follow-up[102-104].

Malnutrition, which is associated with the pathogenesis of hepatolithiasis[3], is also associated with sarcopenia and obesity. Sarcopenia is a prognostic marker in chronic diseases, such as liver failure[105], as well as an independent risk factor for poor prognosis in malignancies[106]. In a large Chinese multicentre cohort, sarcopenia and sarcopenic obesity were associated with worse short-term outcomes after hepatectomy for hepatolithiasis. Patients with sarcopenia had a more difficult perioperative recovery period and a longer hospital stay ($P < 0.001$), along with higher rates of pneumonia ($P = 0.017$), bile leakage ($P = 0.03$), and rate of intensive care unit requirement ($P = 0.004$). Sarcopenic and obese patients had considerably worst intra- and post-operative outcomes, with a higher rate of blood loss ($P < 0.001$), longer operation time ($P < 0.001$), longer hospital stay ($P < 0.001$) and a higher incidence of post-operative major complications ($P = 0.024$) [107].

Dong, Tsunoda and LHO are the classification systems which also suggest the best treatment plan depending on the characteristics of hepatic and biliary involvement. For patients with localised disease and those with diffuse disease with parenchymal atrophy, the Dong and LHO criteria agree that the best outcomes are with hepatectomy. Nevertheless, it is important to take into consideration the Zurich criteria[69] for safe limits of resectability, which highlight that in the presence of normal liver function, the future liver remnant should be at least 30% of the total liver volume, while in cirrhotic patients with Child–Pugh grade A but without portal hypertension, this should be at least 50% of the total liver volume. Portal hypertension or Child–Pugh grade B/C are considered prohibitive for consideration of hepatectomy. Patients that could not tolerate an extensive hepatic resection should be treated with palliative lithotomy and hepatectomy of the smallest area possible. Some of these cases should be discussed with the transplant team to assess feasibility of OLT.

For patients that have concurrent biliary strictures and/or features associated with recurrent cholangitis and bile stasis, the classification systems have different recommendations. The Tsunoda classification argues that there is no difference in outcomes between different biliary reconstruction approaches (*i.e.*, papilloplasty, hepaticoduodenostomy or hepaticojejunostomy) in patients with localised disease. However, this system discourages the use of choledochoduodenostomy and papilloplasty in cases with diffuse disease affecting both hepatic lobes due to the risk of ascending cholangitis. In these cases, Dong's classification advocates for a hepaticojejunostomy. The LHO classification system, on the other hand, suggests that cases of intrahepatic strictures should be treated with hepatectomy of the affected segment. For hilar strictures, it suggests cholangiolithotomy and strictureplasty, leaving hepaticojejunostomy or choledochojejunostomy reserved to cases where the sphincter of Oddi is compromised.

In cases where hepatolithiasis has been complicated by development of intrahepatic CCA, curative-intent treatment can be pursued in the absence of metastatic disease, in the form of radical hepatic resection with or without lymphadenectomy, depending on individual circumstances[108-110]. Depending on the location and size of the tumour, the extend of the required hepatectomy varies from minor to major and, of course the Zurich criteria[66] need to be taken into account, with particular attention to the state of the future liver remnant, in the context of hepatolithiasis.

### Liver transplantation

As long-standing hepatolithiasis may be characterised by repeated episodes of cholangitis, liver abscesses and liver atrophy, it can consequentially lead to secondary biliary cirrhosis with portal hypertension and liver failure[49,111]. In

this setting, OLT is an option, even though relevant experience is scarce, with no more than 30 cases reported[49,111-113]. However, all patients transplanted for hepatolithiasis had favourable long-term outcomes, with the majority having developed secondary biliary cirrhosis prior to OLT[49,111-113]. Postoperative complications were not uncommon, but no mortality has been recorded[49,111-113]. In 2008, Chen *et al*[112] published their experience with 15 patients who underwent OLT for hepatolithiasis. All recipients had at least one biliary operation prior to OLT. Hepaticojejunostomy was used in all 15 cases for biliary reconstruction. All recipients had significantly improved health status, disability, and psychological wellness at 1-year post-transplant, and improved quality of life overall. Based on their successful experience, Chen *et al*[112] proposed two categories of patients with hepatolithiasis suitable for treatment with OLT: (1) Those with decompensated secondary biliary cirrhosis with liver failure; and (2) those with compensated secondary biliary cirrhosis or even absence of cirrhosis, with frequent episodes of cholangitis and diffuse bilateral intrahepatic calculi that are not suitable for treatment with hepatectomy, hepaticojejunostomy, and choledochoscopic intervention.

Notably, it must be mentioned that even though OLT is largely considered contraindicated for the management of intrahepatic CCA, in recent years, there has been a number of studies with excellent outcomes. Consequently, the European Network for the study of CCA issued a consensus statement recommendation that OLT should be considered especially in patients with very early stage unresectable intrahepatic CCA (≤ 2 cm) and underlying cirrhosis, as a potentially curative option[109,114]. It appears plausible that such cases may indeed arise in the context of hepatolithiasis.

## PROGNOSIS

Particularly when hepatolithiasis becomes a recurrently symptomatic disease, clinicians need appropriate strategies to assess prognosis and choose the adequate treatment for these patients. Suzuki *et al*[115] used a Japanese cohort followed up for 18 years, to develop a severity classification system for hepatolithiasis. The authors identified liver cirrhosis, intrahepatic CCA, age ≥ 65 years and jaundice occurring for ≥ 1 wk during follow-up as independent prognostic factors in patients with hepatolithiasis. The group then divided these risk factors in major (including intrahepatic CCA and cirrhosis), and minor (including age ≥ 65 years and jaundice at ≥ 1 wk during follow-up). Their system considers these four criteria and divides patients into three groups according to the presence of these factors (*i.e.*, Grade 1 - no factors, Grade 2 - only minor factors, one or both, Grade 3 - one or both major factors). The 5-year survival for patients considered Grade 1 is 97.6%, while it drops to 89.2% and 57.1% for Grade 2 and Grade 3, respectively. Pu *et al*[100] recently created a nomogram with good accuracy to predict the prognosis of patients with recurrent hepatolithiasis after biliary surgery. The variables used in this algorithm include previous surgery for hepatolithiasis, bilateral intrahepatic stones, lack of immediate clearance after operation, neutrophil to lymphocyte ratio ≥ 2.462 and albumin to globulin ratio ≤ 1.5, as independent risk factors for poor prognosis. This nomogram demonstrated superior accuracy than previous models used for prognosis assessment.

Deng *et al*[116], in a prospective cohort study, divided 121 patients who underwent liver resection for intrahepatic CCA into four groups, namely sarcopenia and hepatolithiasis (S-HL), sarcopenia without hepatolithiasis (S-NHL), non-sarcopenia with hepatolithiasis (NS-HL) and non-sarcopenia without hepatolithiasis (NS-NHL). They observed that the first group had the worse prognosis with overall survival 11.5 months ($P < 0.001$). The assessment of recurrence-free survival detected significant differences between the S-HL group and the NS-HL group ($P = 0.005$), and between the S-HL group and the NS-NHL group ($P < 0.001$), both in favour of the absence of sarcopenia, but no significant difference between the S-HL group and the S-NHL group ($P = 0.054$). Authors also identified age, hepatolithiasis, psoas muscle index, and diabetes as independent prognostic factors for overall survival, while age, hepatolithiasis and psoas muscle index are independent prognostic factors for recurrence-free survival.

Nevertheless, these prognostic factors and risk-stratification system require validation in larger and international cohorts.

## CONCLUSION

Hepatolithiasis seems to be dependent on environmental, rather than genetic and ethnic factors. Its clinical manifestations involve a wide spectrum and severity, and complex cases can be challenging to treat. Such cases require input from a multidisciplinary team of hepatologists, endoscopists, interventional radiologists and hepatobiliary surgeons. Classification systems aim to provide useful insight of clinically relevant aspects and guidance for treatment but can be occasionally complex and require further validation. Accumulating expertise with endoscopic and interventional radiology techniques may further expand their application in treating a greater number and more challenging cases. However, the most complex cases require surgical input, alone or in combination with interventional approaches. Surgical or combined treatment should be aggressive and aim, where possible, to remove all affected segments and intrahepatic stones, to reduce the risk of recurrence and development of CCA.

## FOOTNOTES

**Author contributions:** Mavroeidis VK and Saffioti F contributed equally to this work; Mavroeidis VK and Saffioti F conceptualised and designed the study, created the artwork, supervised, and made critical revisions; Motta RV conducted the literature review, did the

analysis, interpretation of data and drafted the original manuscript; all authors prepared the draft and approved the submitted version.

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**Country/Territory of origin:** United Kingdom

**ORCID number:** Rodrigo V Motta 0000-0002-6633-9133; Francesca Saffioti 0000-0001-7635-9931; Vasileios K Mavroeidis 0000-0002-8188-3575.

**Corresponding Author's Membership in Professional Societies:** General Medical Council (UK), 7451513; Royal College of Surgeons of England, 9092145; American College of Surgeons, 03340060; International College of Surgeons, M21313; Faculty of Surgical Trainers of Edinburgh, Royal College of Surgeons of Edinburgh, 188646; Hellenic Surgical Society, 1974; Athens Medical Association, 061331; Institute of Clinical Research, 002934; and Society of Surgical Oncology, 132496.

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*MINIREVIEWS*

# History of chronic gastritis: How our perceptions have changed

Dmitry Bordin, Maria Livzan

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**Dmitry Bordin,** Department of Pancreatic, Biliary and Upper GI Tract Diseases, A.S. Loginov Moscow Clinical Scientific Center, Moscow 111123, Russia

**Dmitry Bordin,** Department of Propaedeutic of Internal Diseases and Gastroenterology, Russian University of Medicine, Moscow 127006, Russia

**Dmitry Bordin,** Department of Outpatient Therapy and Family Medicine, Tver State Medical University, Tver 170100, Russia

**Maria Livzan,** Department of Internal Medicine and Gastroenterology, Omsk State Medical University, Omsk 644099, Russia

**Corresponding author:** Maria Livzan, DSc, Professor, Department of Internal Medicine and Gastroenterology, Omsk State Medical University, No. 12, Lenina Street, Omsk 644099, Russia. mlivzan@yandex.ru

## Abstract

Currently, the diagnostic strategy for chronic gastritis (CG) is aimed not just at fixing the presence of gastric mucosal inflammation, but also at gastric cancer (GC) risk stratification in a particular patient. Modern classification approach with the definition of the stage of gastritis determines the need, activities and frequency of dynamic monitoring of a patient. However, this attitude to the patient suffering from CG was far from always. The present publication is a literature review describing the key milestones in the history of CG research, from the description of the first observations of inflammation of the gastric mucosa, assessment of gastritis as a predominantly functional disease, to the advent of endoscopy of the upper digestive tract and diagnostic gastric biopsy, assessment of the role of *Helicobacter pylori* infection in progression of inflammatory changes to atrophy, intestinal metaplasia, dysplasia and GC.

**Key Words:** Chronic gastritis; Intestinal metaplasia; Dysplasia; Gastric cancer; *Helicobacter pylori*

**Core Tip:** For more than a century, physicians have noted the relationship of chronic gastritis (CG) with the development of gastric cancer, which prompted great interest in the study and systematization of CG in order to better understand the prognosis and develop approaches for cancer prevention. The accumulated knowledge about the etiology, pathogenesis and morphology of gastritis has made it possible to coordinate the general ideas about gastritis in the classifications used by practicing physicians today.

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## INTRODUCTION

The history of studying chronic gastritis (CG) is the history of Nobel Prize-winning scientific discoveries and their implementation in real clinical practice, the history of the emergence and development of endoscopic examination of the stomach and related histopathological findings in gastric mucosa (GM) biopsy material, the formation and realization of predictive approach in medicine in terms of gastric cancer (GC) prognosis based on the assessment of precancerous changes in the GM. Only less than a century ago the idea of CG was formed on the basis of existing diagnostic capabilities of that time: "The subject of gastritis is one very difficult to approach because the scientific physician feels that nothing is known of its etiology, its morbid anatomy is obscure, symptoms are often completely absent, the prognosis is doubtful and, in fact, the one thing about which we can speak with certitude is the treatment there is none"[1]. It was due to the emergence of new methods of studying the function of the stomach and the structure of its mucosa that new knowledge about various etiologic factors of gastritis, the peculiarities of its course, clinical manifestations and prognosis, approaches to classification were developed, international interdisciplinary expert communities were formed with the adoption of agreements and consensus to determine the most effective tactics of patient management.

## MAIN PART

The first mention of CG is found in the works of German physician Stahl[2] in "Collegium practicum" in 1732, who noted that some febrile diseases are associated with superficial gastric irritation and ulcerative tendencies[3]. However, the history of the term CG has its origins in the work of Broussais[4], a physician in the French Republican Army. In his book "History of chronic phlegmoses or inflammations" (1808), Broussais described common inflammations in the stomach found in almost every deceased soldier, calling them "gastritides" and singled out gastritis (gastritides) as a separate nosologic form[4]. However, his assumptions that these inflammatory changes were formed during life were later refuted by the Scottish professor of pathology Carswell[5] (1838), indicating that the gastric changes described by Broussais were the result of postmortem changes.

The first microscopic description of inflammation of the GM belongs to Charles H. Jones and Edward H. Sieveking (1854)[6] and Wilson Fox[7] (1858), who distinguished diffuse and segmental form of the lesion. Later, the British physician Brinton[8] (1859) in his book "Diseases of Stomach" subdivided the lesion of GM into acute, subacute and chronic, presented their histological differences and compared them with clinical manifestations.

In 1868, the German therapist Kussmaul proposed the use of a gastric tube, which gave rise to the period of studies of gastric functions, including the study of its motility and secretory activity of glands[9]. For the first time there were statements in favor of functional disorders, more and more often gastritis was identified with dyspepsia[10]. Despite this, Fenwick[11] (1880) suggested that CG can rightly be considered an organic pathology, and the presence of pernicious anemia in patients of this group is probably explained by gastric gland atrophy.

In 1904, Faber and Bloch[12], continuing the works of Fenwick, described in detail atrophic changes in the GI tract in a patient with pernicious anemia, indicating their possible relationship. Later Whipple *et al*[13], who at that time studied the role of the liver in hematopoiesis, found that raw liver leads to an increase in the level of erythrocytes in the blood in dogs with posthemorrhagic anemia. In 1926, Minot and Murphy[14], having learned about the discovery of Whipple *et al* [13], applied raw liver to treat pernicious anemia in humans. For their discovery in 1934, Whipple, Minot and Murphy were awarded the Nobel Prize in Physiology and Medicine[15]. In 1948, biochemists Smith[16] and Rickes[17] isolated vitamin B12 from the liver, which gave a new impetus to the study of the causes of this vitamin deficiency in GM atrophy.

At the beginning of the 20$^{th}$ century independently of each other two scientists Faber and Konjetzny made a serious attempt to prove the presence of a morphologic substrate in CG[18]. Faber by introducing 10% formalin solution into the abdominal cavity protected GM from postmortem autolysis and putrefaction. Konjetzny[19] studied resected stomachs in patients with peptic ulcer disease (PUD) and GC by developing a special technique that prevented the possibility of postmortem autolysis of tissue. In his works Konjetzny[19] wrote: "...Ulcer and GC can develop against the background of silent inflammation of the GI tract. We can not distinguish between gastritis, on the background of which appears GC. If we can prevent the development of gastritis or treat it, we can prevent the formation of ulcers and the development of GC. Prevention of gastritis is prevention of ulcers and stomach cancer...". In 1944, Warren and Meissner[20] published

data on a groundbreaking change that can be found in patients with gastritis – intestinal metaplasia (IM).

*In vivo* diagnosis of gastritis became possible after the invention of the semi-rigid gastroscope by Schindler in 1932. In his monograph "Gastritis", he classified gastritis into acute and chronic, subdividing the latter into superficial, atrophic and hypertrophic (1947)[21,22]. Since the time of Schindler, the term "superficial gastritis" was included in the medical dictionary and is still used today to denote non-atrophic gastritis.

In 1949, Wood *et al*[23] reported the invention of a simple biopsy tube, and soon (1957) Hirschowitz[24], a flexible fiber optic fibrogastroscope, which made it possible to perform targeted forceps biopsy under visual control from any part of the stomach. The emergence of this method can rightly be considered a revolutionary event in gastroenterology. In a short time it became universally recognized and widespread, expanding the range of possibilities in the diagnosis of various gastric diseases. Since that time, a new chapter in the history of CG research begins.

In 1956, Cheli and Dodero[25] proposed to classify gastritis into "superficial", "interstitial" and "atrophic" ones. In 1959, Wood and Taft[26] outlined possible etiologic factors of CG: alcohol, diet, stress, radiation and other. A little later, in the 60s of the last century with the help of immunologic studies it was possible to detect in some CG patients the presence of autoantibodies to parietal cells of gastric glands and to Castle's intrinsic factor, which allowed to explain the pathogenesis of autoimmune CG and its connection with vitamin B12 deficiency[27].

In 1972, Whitehead *et al*[28] divided CG topographically into antral, fundal, cardiac, and pyloric, and proposed the division of CG into "active" and "inactive" based on the presence of inflammatory infiltration of the GM and introduced the evaluation of intestinal and pseudopyloric metaplasia into the routine practice of pathologists. A year later, Strickland and MacKay[29] proposed to supplement the classification of CG with etiologic data. They used the terms "type A gastritis" (autoimmune) to denote gastritis of the stomach body, and "type B gastritis" (non-autoimmune) to denote antral gastritis, presumably caused by duodeno-gastric reflux. In 1975, Jerzy Glass and Pitchumoni[30] added "type AB gastritis" to the classification to denote CG extended from the body of the stomach to the prepyloric region.

Another truly revolutionary event was the publication in "*The Lancet*" of an article by Correa *et al*[31,32] where he presented a sequence of pathologic changes in GM (P. Correa's cascade) from the formation of non-atrophic CG and slow, over 20-25 years, development and progression of atrophy (at a rate of 0%-3%-3% per year) to the appearance of specialized intestinal-type epithelium, and then to dysplasia/intraepithelial neoplasia and GC.

The discovery of the bacterium *Helicobacter pylori* (*H. pylori*) was a revolutionary event in gastroenterology and led to a rethinking of existing approaches to the diagnosis and treatment of gastric diseases. The presence of *H. pylori* in the human GM was described more than a century ago, but it was not until the end of the 20th century that the role of the bacterium as a leading etiological factor in CG, PUD, GC, and MALT lymphoma was recognized[33-35]. For a long time, there was a belief that due to low pH values in the stomach, microbial growth and reproduction were impossible, so many researchers thought that the bacteria they detected were accidental representatives of the oral microbiota that had nothing to do with the stomach itself[36].

It is believed that the first to discover colonies of spiral-shaped bacteria in the bottom of dog gastric ulcers were German bacteriologist Bottcher and his colleague Letulle in 1875, and they first suggested that bacteria are the cause of gastric ulcers[37]. However, the bacteria discovered by them were not cultured on the known nutrient media of that time, due to which their hypothesis was criticized and was not further developed, even though later in 1881 the pathologist Klebs[38] reported a bacillus-like organism found in the lumen of the gastric glands and in the GM of dogs with the formation of a characteristic "inflammatory infiltration". And although Klebs noted only the presence of concomitant inflammatory infiltration without making specific conclusions, this report is considered by many researchers as the first description of gastritis caused by *H. pylori*.

In 1889, Jaworski, professor of medicine at the Jagiellonian University in Krakow (Poland), examined stomach flushes obtained from humans and discovered a characteristic spiral-shaped bacteria, which he called *Vibrio Rugula*[39]. Jaworski suggested that *Vibrio Rugula* may play a possible pathogenic role in the development of gastric diseases. The work of Jaworski were published in Polish, and perhaps that is why they were not widely disseminated and recognized.

In 1893, an Italian researcher, famous anatomist Bizzozero together with his student Camillo Golgi described spiral-shaped bacteria in the parietal cells and glands of the GM of dogs, later identified as *H. canis, H. Felis* and *H. heilmannii*. Bizzozero[40] noted that these microorganisms could infect the mucosa of both the pyloric and fundal parts of the stomach. Three years later, in 1896, in an article entitled "Spirillum of the mammalian stomach and its behavior with respect to the parietal cells". Salomon[41] reported the presence of spirochetes in the GM of dogs, cats, and rats and described a series of experiments where he managed to transfer the spirochete bacterium detected in the stomach of dogs to white mice.

In 1906, Krienitz[42] identified spirochetes in the stomach of a patient with carcinoma. 9 years later, similar bacteria were found in patients with gastric and duodenal ulcers. Around the same time, the presence of urease activity in the human stomach was documented, but it was thought to occur directly in mucosal cells and was not related to the presence and activity of bacteria[43].

In 1923, the scientist Edkins[44] from London, who had previously gained fame for the discovery of the hormone gastrin, using Giemsa staining identified spiral-shaped bacteria in the bottom of gastric ulcers, as well as in the antral part of the stomach and put forward a theory about the relationship between the development of PUD and the bacterium he discovered, which he named *Spirochete regaudi*.

In 1938 in the United States Doenges[45] found spiral-shaped bacteria in the GM of the rhesus macaque he studied and in 43% of resected human stomach samples.

In 1974, Ito and Schofield[46] from Harvard Medical School (United States), who made the first anatomical description of the GM under an electron microscope, published a photograph of the gastric parietal cell, which showed a bacterium later identified as *H. pylori*.

The coccoid and vegetative bacterial forms later identified also as H. pylori were discovered in Russia in 1974 by I.A. Morozov and L.I. Aruin during electron microscopic observation of gastric mucosa sections from patients after proximal vagotomy in connection with peptic ulcer[47].

In 1979, during a routine histologic examination, Warren, a pathologist at the Royal Perth Hospital (Australia), noticed a blue line on the surface of the GM of a patient with active CG. After analyzing a large volume of biopsy material, he assumed that these were bacteria that somehow played a role in gastric disease. However, in light of the prevailing dogma at the time about hydrochloric acid-induced ulcers and the skepticism of his colleagues, Warren was reluctant to discuss this controversial observation in the wider gastroenterologic community[48]. Coincidentally, at this time, a young research fellow gastroenterologist, Marshall was looking for a project to complete his internship at the Royal Perth Hospital, he was attracted to the hypothesis of Warren and they embarked on a collaborative research journey to explore ways of culturing *Unidentified bacilli*[49]. Attempts to grow the bacterium were unsuccessful until April 1982. This event occurred by chance, when cultures were left in the thermostat over the long Easter weekend and bacterial colonies appeared on the 5$^{th}$ day after biopsy. Among the first 135 patients studied by Warren and Marshall, nearly all were diagnosed with gastritis, and more than 65% were found to be infected with the bacterium. The microorganism was found in all 13 patients with duodenal ulcers and in 18 of 22 patients with gastric ulcers. In 1982, Marshall submitted a summary detailing his observations to the Australian Gastroenterological Association, where he was categorically rejected. However, on October 22, 1982, he managed to report his work at the International Workshop on Campylobacter infections, and later in 1983 these data were published as 2 Letters in "*The Lancet*"[50]. The microorganism was initially named *Campylobacter pyloridis* because of its similarity to other *Campylobacter* in morphology and structure.

In order to establish the causal relationship between infection and gastritis, Marshall ventured into a self-infection experiment. In 1984, after a preliminary endoscopy, the results of which showed that *Campylobacter pyloridis* was absent in the scientist's stomach, Marshall intentionally infected himself with *Campylobacter pyloridis* by drinking the contents of a petri dish in which he had cultured the microorganism, and on the 7$^{th}$ day he woke up with severe nausea and vomiting, and on the 10$^{th}$ day he underwent a repeat endoscopy and biopsy, which showed massive inflammation, and a culture revealed *Campylobacter pyloridis*. On the 14$^{th}$ day of the experiment, the physician started therapy with bismuth salts and metronidazole for 14 d, after which the complaints disappeared. At the control endoscopy one month after the completion of treatment, the histology returned to normal, and the bacteria disappeared[51].

Further research allowed scientists to accumulate a sufficient database proving the undeniable role of the bacterium in the development of gastritis and PUD. Subsequently, it was shown that *Campylobacter pyloridis* does not belong to the genus *Campylobacter*, and in 1989 the bacterium received its current name *H. pylori*.

In October 1987, in Copenhagen, Denmark, to promote interdisciplinary research into the pathogenesis of *H. pylori*-associated diseases The European *Helicobacter pylori* Study Group (EHSG) was founded with the active participation of Peter Malfertheiner and Francis Megraud. And this has been followed by an exponential increase in the number of studies on the role of bacteria in the development of gastric diseases. Since then, the EHSG has organized a series of consensus conferences with leading experts to develop approaches to the diagnosis and treatment of infection based on evidence-based medicine standards[52].

For the "discovery of *H. pylori* and elucidation of its role in the development of gastritis and peptic ulcer disease" Australian scientists Barry Marshall and Robin Warren in 2005, the Nobel Assembly of the Karolinska Institute in Stockholm awarded the prize in the field of medicine and physiology[49].

In parallel with the study of the role of *H. pylori* as a leading etiologic factor of gastritis, other possible causes of the disease were also evaluated. In 1988, Wyatt and Dixon[53] proposed to use the term "gastritis due to duodenogastric reflux". Dixon proposed to use the term "type C gastritis" or "chemical gastritis". However, several years later it was shown that most cases of chemical gastritis in the intact (unoperated) stomach were caused not by bile reflux but by the intake of nonsteroidal anti-inflammatory drugs, after which "type C gastritis" or "chemical gastritis" was used for a long time to describe inflammatory changes of the GM caused by both etiologic factors[54].

At the International Congress of Gastroenterology held in Sydney (1990), the "Sydney system" of gastritis classification was adopted[55], where it was proposed to classify gastritis according to three characteristics, presented by analogy with morpheme parsing of a word, where the "prefix" means the etiology of gastritis, the "root" - topography (stomach body, antral section), the "suffix" - morphological characteristics (degree of inflammation activity, severity of inflammation, severity of atrophy and metaplasia, presence and degree of colonization by *H. pylori*)[56]. The system also established a four-level scale to determine the severity of histopathological changes. It should be noted that the new classification was criticized because some of the frequently used descriptive names such as "multifocal atrophic gastritis" or "diffuse antral gastritis" were not included in the system[57,58].

Later, Houston (1994) revised the Sydney classification, restored the division of CG into types A, B, and C, and added drug-induced CG to the chemical gastritis (type C) section. Among other things, this updated system introduced a visual analog scale to assess the severity of histopathologic elements, and a protocol for taking biopsy specimens during endoscopic examination was proposed[55].

In 2005, international experts gastroenterologists and pathologists developed a system to determine the stage of CG - Operative Link for Gastritis Assessment (OLGA)-system[59]. It was based on the biopsy sampling protocol defined in Houston (1994): Two biopsy specimens from the antral part of the stomach at a distance of 2-3 cm from the gatekeeper along the lesser and greater curvature, one biopsy specimen from the gastric angle and two biopsy specimens from the stomach body at a distance of 8 cm from the cardia rosette along the lesser and greater curvature[60].

The purpose of the established OLGA system is to translate histopathological data into a standardized report with information about the state of the stomach (topography and degree of atrophic changes) and to divide patients according to the risk of GC. In 2008, based on the results of OLGA system application, a number of papers appeared in which it was convincingly demonstrated that patients with severe atrophy (OLGA III and IV) have a higher risk of developing GC.

**Table 1 Etiological classification of gastritis presented in the ICD XI[63]**

| Etiological group of gastritis | Subgroup | Note |
|---|---|---|
| I. Autoimmune CG | | Etiology unknown, autoimmune pathogenesis |
| II. Infectious CG | Induced by *H. pylori* | |
| | Bacterial non-helicobacter | Caused by enterococci |
| | | Caused by mycobacteria |
| | | Caused by Treponema pallidum |
| | Viral | Caused by enterovirus |
| | | Caused by cytomegalovirus |
| | Fungal | For gastric mucormycosis |
| | | For gastric candidiasis |
| | | For gastric histoplasmosis |
| | Parasitic | Caused by Cryptosporidium |
| | | Caused by strongyloidiasis |
| | | Caused by anisokiasis |
| III. Caused by external causes | Drug-induced gastritis | |
| | Alcoholic | |
| | Radiation | |
| | Chemical | |
| | Caused by biliary reflux | |
| | Caused by other specified external causes | |
| IV. Caused by special reasons | Lymphocytic | |
| | Ménétrier's disease | |
| | Allergic | |
| | Eosinophilic | |
| V. Gastritis due to other classified diseases | For sarcoidosis | |
| | For vasculitis | |
| | For Crohn's disease | |

CG: Chronic gastritis.

This allowed us to conclude that patients with atrophy stages III-IV belong to a high-risk group for the development of GC and require different approaches to curation, as well as dynamic monitoring[61,62].

Along with the OLGA system, the Operative Link on Gastritic Intestinal Metaplasia assessment (OLGIM) system for evaluation of CG stage has also entered clinical practice. In the modified OLGIM system atrophy assessment is based on the consideration of IM only as an indicator of CG stage, because IM is the most reproducible criterion of atrophy[63,64]. The question of comparing these classifications from the point of view of sensitivity is still debatable, since the OLGIM, which is more reproducible in practical application, may underestimate the true severity of absolute (nonmetaplastic) atrophy[65]. Modifications of these classifications are based on similar principles of integral assessment[66].

In 2015, an international meeting of experts in the field of gastroenterology was held in Kyoto (Japan)[67]. During the conference, the data on etiological factors of CG were systematized, which were later fully transferred to ICD XI[68]. The etiologic classification of gastritis reflected in ICD XI is presented in Table 1.

The most relevant topics related to inflammatory gastric diseases were addressed in the work of the initiative group - the Real-world Gastritis Initiative, or RE.GA.IN. - from redefining the disease to clinical diagnosis and assessing prognosis[69]. After lively debates on the most controversial aspects, the RE. GA.IN. consensus summarized existing published and additional scientific evidence to produce patient-centered, evidence-based statements that will help health professionals in their actual clinical practice.

## CONCLUSION

Thus, the problem of CG has been discussed by the medical community for about 300 years (Supplementary Table 1). Historically, this diagnosis did not immediately gain the right to exist. For a long time it was attributed to the number of functional diseases, thus slowing down the detailed study of CG. The data accumulated to date allow not only to detect changes in the GM, but also to predict the course of CG, and the progress achieved in the field of molecular biology and genetic engineering opens new opportunities for early diagnosis and prevention of GC.

## FOOTNOTES

**Author contributions:** Bordin D and Livzan M were designed the outline of the paper, performed writing of the paper; Livzan M prepared the tables; Bordin D coordinated the writing of the paper.

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**Country/Territory of origin:** Russia

**ORCID number:** Dmitry Bordin 0000-0003-2815-3992; Maria Livzan 0000-0002-6581-7017.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Bayesian network-based survival prediction model for patients having undergone post-transjugular intrahepatic portosystemic shunt for portal hypertension

Rong Chen, Ling Luo, Yun-Zhi Zhang, Zhen Liu, An-Lin Liu, Yi-Wen Zhang

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**Rong Chen, Ling Luo, Yun-Zhi Zhang, Zhen Liu, An-Lin Liu, Yi-Wen Zhang,** Department of Infectious Diseases, Second Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

**Corresponding author:** Ling Luo, MNurs, Researcher, Department of Infectious Diseases, Second Affiliated Hospital of Chongqing Medical University, No. 288 Tianwen Avenue, Nanan District, Chongqing 400016, China. ll7765@cqmu.edu.cn

## Abstract

### *BACKGROUND*

Portal hypertension (PHT), primarily induced by cirrhosis, manifests severe symptoms impacting patient survival. Although transjugular intrahepatic portosystemic shunt (TIPS) is a critical intervention for managing PHT, it carries risks like hepatic encephalopathy, thus affecting patient survival prognosis. To our knowledge, existing prognostic models for post-TIPS survival in patients with PHT fail to account for the interplay among and collective impact of various prognostic factors on outcomes. Consequently, the development of an innovative modeling approach is essential to address this limitation.

### *AIM*

To develop and validate a Bayesian network (BN)-based survival prediction model for patients with cirrhosis-induced PHT having undergone TIPS.

### *METHODS*

The clinical data of 393 patients with cirrhosis-induced PHT who underwent TIPS surgery at the Second Affiliated Hospital of Chongqing Medical University between January 2015 and May 2022 were retrospectively analyzed. Variables were selected using Cox and least absolute shrinkage and selection operator regression methods, and a BN-based model was established and evaluated to predict survival in patients having undergone TIPS surgery for PHT.

### *RESULTS*

Variable selection revealed the following as key factors impacting survival: age, ascites, hypertension, indications for TIPS, postoperative portal vein pressure (post-PVP), aspartate aminotransferase, alkaline phosphatase, total bilirubin, prealbumin, the Child-Pugh grade, and the model for end-stage liver disease

(MELD) score. Based on the above-mentioned variables, a BN-based 2-year survival prognostic prediction model was constructed, which identified the following factors to be directly linked to the survival time: age, ascites, indications for TIPS, concurrent hypertension, post-PVP, the Child-Pugh grade, and the MELD score. The Bayesian information criterion was 3589.04, and 10-fold cross-validation indicated an average log-likelihood loss of 5.55 with a standard deviation of 0.16. The model's accuracy, precision, recall, and F1 score were 0.90, 0.92, 0.97, and 0.95 respectively, with the area under the receiver operating characteristic curve being 0.72.

*CONCLUSION*

This study successfully developed a BN-based survival prediction model with good predictive capabilities. It offers valuable insights for treatment strategies and prognostic evaluations in patients having undergone TIPS surgery for PHT.

**Key Words:** Bayesian network; Cirrhosis; Portal hypertension; Transjugular intrahepatic portosystemic shunt; Survival prediction model



**Core Tip:** This study introduces an advanced Bayesian network model to better understand the interrelationships among prognostic factors and their combined impact on prognosis, thus enhancing the accuracy of survival predictions for patients with cirrhosis-induced portal hypertension after the transjugular intrahepatic portosystemic shunt procedure. This approach potentially assists in refining and advancing current prognostic research.

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## INTRODUCTION

Portal hypertension (PHT) is primarily characterized by a significant increase in venous pressure within the portal venous system. This condition often results in severe symptoms like refractory ascites and bleeding from esophageal and gastric varices, which exacerbate disease progression and profoundly impact survival prognosis[1]. Cirrhosis accounts for approximately 90% of PHT cases[2]. The transjugular intrahepatic portosystemic shunt (TIPS) procedure represents a crucial intervention in effectively managing PHT and its complications[3]. However, studies indicate that TIPS, while effective in lowering the portal vein pressure (PVP), may lead to complications like hepatic encephalopathy and stent-related dysfunctions, thus influencing overall patient survival rates[4,5]. Therefore, it is crucial to actively identify and thoroughly analyze the prognostic factors and their interrelations after TIPS in PHT.

Previous research on prognostic factors for TIPS in PHT has shown that preoperative factors, including total bilirubin (TBIL) levels, serum creatinine (SCr), and sarcopenia, are correlated with patient survival after TIPS surgery[3,6,7]. Recent multivariate survival analyses, such as those by Huang *et al*[8], have used logistic regression to identify clinical variables that significantly affect post-TIPS outcomes in patients with cirrhosis, resulting in the creation of a support vector machine model focused on liver disease-related mortality. Sun *et al*[9] used a random forest algorithm in combination with variables including age and Child-Pugh scores to develop a model for predicting disease-free survival in cirrhotic patients having PHT who were treated with TIPS. Li *et al*[10] assessed the 5-year survival rates in Chinese patients with cirrhosis undergoing TIPS placement using Viatorr® stents, identified post-TIPS overt hepatic encephalopathy (OHE) and Child-Pugh grade as independent prognostic factors for mortality, and developed a nomogram-based predictive model for post-TIPS OHE. However, these studies did not explore the interrelationships and mechanisms of prognostic factors. Consequently, using more advanced modeling techniques to deeply investigate the interactions among these factors is crucial.

Bayesian networks (BNs), based on the Bayesian theorem, are a type of machine learning algorithm. They combine prior knowledge with data, illustrate variable relationships through directed acyclic graphs, and elucidate node connections using conditional probabilities[11]. By integrating probability and graph theories, BNs effectively demonstrate the interactions among independent variables and their complex relationships with dependent variables in factor analysis[12]. Consequently, BNs are a powerful tool for predictive, classificatory, and causal analyses in data mining. Recently, BNs have been extensively applied in risk assessment and prognosis prediction for various diseases. For example, Li *et al*[13] used several factors, such as age, carcinoembryonic antigen, and carbohydrate antigen 19-9, to develop a BN model predicting the prognosis of rectal cancer and achieved an area under the receiver operating characteristic curve (AUC) of 0.801. Similarly, Wu *et al*[14] used parameters like age, pathological grade, and liver infiltration to develop a BN model for predicting survival in gallbladder cancer patients after radical cholecystectomy. This model

showed internal and external validation AUCs of 0.757 and 0.765, respectively, which exceeded the accuracy of traditional nomograms. However, to our knowledge, no research yet has used BNs to analyze the prognosis of patients having undergone TIPS surgery for PHT.

In this study, we focus on comprehensive analysis of factors affecting post-TIPS prognosis in patients with PHT, using BN approaches to fully assess preoperative factors. We herein aim to reveal the interdependencies among these factors and to develop a BN model that predicts patient survival after TIPS, thus providing substantial data support for clinical decision-making.

## MATERIALS AND METHODS

### Study participants

In this retrospective study, we collected data of 393 patients with cirrhosis-induced PHT who received their first TIPS treatment at the Second Affiliated Hospital of Chongqing Medical University between January 2015 and May 2022. Follow-up involved outpatient visits, hospital admissions, and phone calls, and we documented all-cause mortality and liver transplants during and after hospitalization until May 2023. The inclusion criteria were as follows: (1) Age ≥ 18 years; (2) meeting the diagnostic criteria for cirrhosis based on the "2019 Cirrhosis Diagnosis and Treatment Guidelines" [1]; (3) showing PHT-related complications like hepatic ascites and variceal hemorrhage; and (4) receiving TIPS treatment for the first time. The exclusion criteria were as follows: (1) Individuals with significant cardiac, pulmonary, or renal conditions; (2) PHT cases not related to cirrhosis; (3) patients with coexisting malignancies; and (4) patients having previously received TIPS or liver transplants.

### Data collection and processing

Data collection included patients' baseline information, disease-related metrics, laboratory results, and overall survival times. Following literature review and expert advice, 34 potential prognostic factors were identified for analysis. These include sex, age, etiology of cirrhosis, diabetes, hypertension, indications for TIPS, the portal vein thrombosis status, endoscopic therapy history, splenectomy history, ascites, preoperative hepatic encephalopathy, spontaneous bacterial peritonitis, type of stent, preoperative PVP (pre-PVP), postoperative PVP (post-PVP), the Child-Pugh grade, the model for end-stage liver disease (MELD) score, and laboratory values such as serum potassium, sodium (Na), SCr, blood urea nitrogen, albumin (ALB), alanine aminotransferase, aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase, TBIL, prealbumin (PA), international normalized ratio, prothrombin time (PT), fibrinogen, hemoglobin, white blood cell count (WBC), and platelets. Missing data were imputed using maximum likelihood estimation, with continuous variables discretized for analysis. Considering varying follow-up durations among patients, 24 months was selected as a key time point, with clinical laboratory indicators discretized based on reference values. Child-Pugh grading was as follows: A (5-6 points), B (7-9 points), and C (10-15 points). MELD scores were as follows: > 18 (high risk), 15-18 (moderate risk), and ≤ 14 (low risk). Optimal cutoffs for patient age, pre-PVP, and post-PVP were determined using the X-tile software (Yale University, V3.6.1).

### Variable selection

Variable selection combined Cox analysis with least absolute shrinkage and selection operator (LASSO) regression. Variables showing an association with the outcome in univariate Cox regression analysis ($P < 0.05$) were then analyzed using LASSO regression to identify significant predictors of prognosis for BN development.

### Statistical analysis

Simple statistical descriptive analyses and survival analyses were performed using IBM SPSS 25 (IBM Corporation, Armonk, NY, United States). All categorical variables were expressed as numbers and percentages. Survival rates were calculated using the Kaplan-Meier method. Univariate analysis was performed through Cox regression; variables with a $P$ value of < 0.05 were considered significant and included in further LASSO regression analysis. The execution of LASSO regression and the generation of survival curve were carried out using the Free Statistics analysis platform (Version 1.9, FreeClinical Medical Technology Co., Ltd., Beijing, China). The bnlearn package in RStudio (version 4.3.1) facilitated the development of BN models using a tabu search algorithm for structural learning, incorporating expert input for whitelist and blacklist settings, and learning network parameters through maximum likelihood estimation. Model performance assessment and validation used the Bayesian information criterion (BIC) and 10-fold cross-validation. Evaluation metrics included accuracy, precision, recall, F1 score, and AUC values. The BN's topological structure and conditional probability inferences were drawn by Netica 32.0 (Norsys Software Corp., Vancouver, BC, Canada).

## RESULTS

### Participant characteristics

The study included 393 patients with PHT, comprising 278 male (70.7%) and 115 female patients (29.3%). The median age at the time of surgery was 51 years (range, 45-58 years), with the predominant cause of cirrhosis being hepatitis B virus infection (64.6%), as detailed in Table 1. During the follow-up period, 68 patients died. The cumulative survival rates at 1, 3, and 5 years were 96.2%, 87.9%, and 76.6%, respectively, as shown in Figure 1.

**Table 1 Baseline characteristics of the study cohort, *n* (%)**

| Variable | | Variable | |
|---|---|---|---|
| Sex | | None | 151 (38.4) |
| Male | 278 (70.7) | Small | 138 (35.1) |
| Female | 115 (29.3) | Moderate to large | 104 (26.5) |
| Age (yr) | | Pre-HE | |
| < 60 | 307 (78.1) | No | 387 (98.5) |
| ≥ 60 | 86 (21.9) | Yes | 6 (1.5) |
| Etiology of cirrhosis | | SBP | |
| Hepatitis B virus | 254 (64.6) | Yes | 106 (27.0) |
| Hepatitis C virus | 16 (4.1) | No | 287 (73.0) |
| Alcoholic | 35 (8.9) | Type of stent | |
| Autoimmune | 40 (10.2) | Composite stent[1] | 265 (67.2) |
| Others | 48 (12.2) | Viatorr® stent | 128 (32.6) |
| Diabetes | | Pre-PVP ($mmH_2O$) | |
| Yes | 67 (17.0) | > 33.9 | 305 (77.6) |
| No | 326 (83.0) | ≤ 33.9 | 88 (22.4) |
| Hypertension | | Post-PVP ($mmH_2O$) | |
| Yes | 27 (6.9) | > 23.1 | 295 (75.1) |
| No | 366 (93.1) | ≤ 23.1 | 98 (24.9) |
| Indications for TIPS | | K (mmol/L) | |
| Variceal bleeding | 357 (90.8) | < 3.5 | 95 (24.2) |
| Refractory ascites | 36 (9.2) | 3.5-5.5 | 295 (75.1) |
| Formation of PVT | | > 5.3 | 3 (0.8) |
| Yes | 94 (23.9) | Na (mmol/L) | |
| No | 299 (76.1) | < 137 | 124 (31.6) |
| Endoscopic treatment history | | ≥ 137 | 269 (68.4) |
| Yes | 115 (29.3) | BUN (mmol/L) | |
| No | 278 (70.7) | < 3.1 | 28 (7.1) |
| Splenectomy history | | ≥ 3.1 | 365 (92.9) |
| Yes | 15 (3.8) | SCr (μmol/L) | |
| No | 378 (96.2) | ≤ 106 | 356 (90.6) |
| Ascites | | > 106 | 37 (9.4) |
| ALB (g/L) | | PT (s) | |
| < 40 | 330 (84.0) | ≤ 14.5 | 54 (13.7) |
| ≥ 40 | 63 (16.0) | > 14.5 | 339 (86.3) |
| ALT (U/L) | 352 (88.0) | FIB (g/L) | |
| ≤ 50 | 350 (89.1) | < 2 | 216 (55.0) |
| > 50 | 43 (10.9) | ≥ 2 | 177 (45.0) |
| AST (U/L) | | Hb (g/L) | |
| ≤ 40 | 279 (71.0) | < 130 | 328 (83.5) |
| > 40 | 114 (29.0) | ≥ 130 | 65 (16.5) |
| ALP (U/L) | | WBC ($10^9$/L) | |

| | | | |
|---|---|---|---|
| ≤ 135 | 342 (87.0) | < 3.5 | 236 (60.1) |
| > 135 | 51 (13) | 3.5-9.5 | 144 (36.6) |
| GGT (U/L) | | > 9.5 | 13 (3.3) |
| ≤ 60 | 295 (75.1) | PLT ($10^9$/L) | |
| > 60 | 98 (24.9) | < 125 | 322 (81.9) |
| TBIL (μmol/L) | | ≥ 125 | 71 (18.1) |
| ≤ 28 | 301 (76.6) | Child-Pugh grade | |
| > 28 | 92 (23.4) | A | 169 (43.0) |
| PA (mg/L) | | B | 185 (47.1) |
| < 150 | 321 (81.7) | C | 39 (9.9) |
| ≥ 150 | 72 (18.3) | MELD score | |
| INR | | Low risk | 354 (90.1) |
| ≤ 1.3 | 190 (48.3) | Medium risk | 31 (7.9) |
| > 1.3 | 203 (51.7) | High risk | 8 (2.0) |

[1]Combined stent: Covered stent + bare stent.

TIPS: Transjugular intrahepatic portosystemic shunt; PVT: Portal vein thrombosis; pre-HE: Preoperative hepatic encephalopathy; SBP: Spontaneous bacterial peritonitis; pre-PVP: Preoperative portal vein pressures; post-PVP: Postoperative portal vein pressures; K: Potassium; Na: Sodium; SCr: Serum creatinine; BUN: Blood urea nitrogen; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; TBIL: Total bilirubin; PA: Prealbumin; INR: International normalized ratio; PT: Prothrombin time; FIB: Fibrinogen; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelets; MELD: Model for end-stage liver disease.

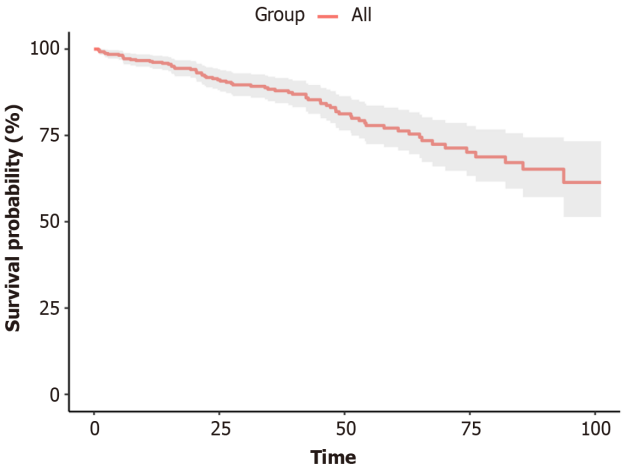


**Figure 1 Overall survival of the study cohort.**

### *Variable selection results*

**Univariate Cox regression analysis:** A univariate Cox regression analysis was conducted on all collected variables potentially affecting post-TIPS survival in patients with PHT. Results indicated that factors of age, etiology of cirrhosis, hypertension, indications for TIPS, ascites, post-PVP, Na, ALB, AST, ALP, TBIL, PA, PT, WBC, the Child-Pugh grade, and the MELD score were associated with overall post-TIPS survival ($P < 0.05$), as shown in Table 2.

**Least absolute shrinkage and selection operator regression analysis:** Factors with a $P$ value of < 0.05 in the univariate Cox regression were subjected to LASSO regression analysis. We selected the optimal lambda value (lambda.min = 0.027). The results indicated that age, ascites, hypertension, indications for TIPS, post-PVP, AST, ALP, TBIL, PA, the Child-Pugh grade, and the MELD score were significantly related to overall patient survival, as shown in Figure 2.

### *BN survival prediction model for patients having undergone TIPS surgery for PHT*

The abovementioned 11 prognostic factors identified by univariate Cox and LASSO regression were incorporated into the BN model, and its topological structure is shown in Figure 3. The BN model in this study illustrated 12 nodes and 17 directed edges, wherein each node signifies a factor influencing the survival time after TIPS, and the directed edges

**Table 2 Univariate survival analysis of patients having undergone transjugular intrahepatic portosystemic shunt surgery for portal hypertension**

| Variable | HR | 95%CI | *P* value | Variable | HR | 95%CI | *P* value |
|---|---|---|---|---|---|---|---|
| Age (yr) | | | | < 40 | 1.00 | | |
| < 60 | 1.00 | | | ≥ 40 | 0.39 | 0.16-0.97 | 0.043 |
| ≥ 60 | 3.64 | 2.24-5.89 | < 0.001 | AST (U/L) | | | |
| Etiology of cirrhosis | | | | ≤ 40 | 1.00 | | |
| Hepatitis B virus | 1.00 | | | > 40 | 1.73 | 1.07-2.81 | 0.026 |
| Hepatitis C virus | 0.85 | 0.20-3.53 | 0.820 | ALP (U/L) | | | |
| Alcoholic | 3.23 | 1.74-5.99 | < 0.001 | ≤ 135 | 1.00 | | |
| Autoimmune | 1.51 | 0.67-3.41 | 0.317 | > 135 | 2.60 | 1.5-4.51 | < 0.001 |
| Others | 1.34 | 0.64-2.78 | 0.434 | TBIL (μmol/L) | | | |
| Hypertension | | | | ≤ 28 | 1.00 | | |
| No | 1.00 | | | > 28 | 2.65 | 1.64-4.29 | < 0.001 |
| Yes | 3.75 | 1.89-7.44 | < 0.001 | PA (mg/L) | | | |
| Indications for TIPS | | | | < 150 | 1.00 | | |
| Variceal bleeding | 1.00 | | | ≥ 150 | 0.38 | 0.16-0.88 | 0.024 |
| Refractory ascites | 2.23 | 1.14-4.36 | 0.020 | WBC ($10^9$/L) | | | |
| Ascites | | | | < 3.5 | 1.00 | | |
| None | 1.00 | | | 3.5-9.5 | 1.70 | 1.04-2.76 | 0.033 |
| Small | 0.61 | 0.31-1.22 | 0.164 | > 9.5 | 1.49 | 0.46-4.88 | 0.507 |
| Moderate to large | 2.51 | 1.48-4.26 | < 0.001 | Child-Pugh grade | | | |
| Post-TIPS PVP ($mmH_2O$) | | | | A | 1.00 | | |
| ≤ 23.1 | 1.00 | | | B | 2.09 | 1.16-3.76 | 0.014 |
| > 23.1 | 2.85 | 1.30-6.23 | 0.009 | C | 5.12 | 2.53-10.37 | < 0.001 |
| Na (mmol/L) | | | | MELD score | | | |
| < 137 | 1.00 | | | Low risk | 1.00 | | |
| ≥ 137 | 0.54 | 0.33-0.89 | 0.016 | Medium risk | 2.33 | 1.18-4.58 | 0.014 |
| ALB (g/L) | | | | High risk | 5.98 | 2.38-15.05 | < 0.001 |

Post-PVP: Postoperative portal vein pressures; Na: Sodium; ALB: Albumin; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin; PA: Prealbumin; WBC: White blood cell count; MELD: Model for end-stage liver disease.

denote probabilistic dependencies among these factors. Percentages in the figure denote the prior probabilities of each node. For example, the prior probability of the survival, denoted as *P* (survival time), was estimated to be 81.2%. Within the BN framework, age, ascites, indications for TIPS, concurrent hypertension, post-PVP, the Child-Pugh grade, and the MELD score were the parent nodes directly linked to the survival time; the remaining variables are indirectly associated with the survival time.

### Model validation and assessment

The BIC produced a score of -3589.04. Ten-fold cross-validation showed an average log-likelihood loss of 5.55 with a standard deviation of 0.16. The model demonstrated an accuracy, precision, recall, and F1 score of 0.90, 0.92, 0.97, and 0.95, respectively, with an AUC value of 0.72.

### Bayesian inference

BNs can infer unknown nodes based on known ones. In cases with moderate to severe ascites, the probability of refractory ascites as a TIPS indication increases to 26.40%, the probability of PA levels being below 150 mg/L stands at 94.20%, the likelihood of post-PVP exceeding 23.1 $mmH_2O$ is at 82.1%, the chance of having Child-Pugh grade C is 22.70%, the probability of age being over 60 years is 33.7%, and the 2-year mortality rate is 28.30%, as shown in Figure 4. In scenarios where patients have concurrent hypertension, are aged ≥ 60 years, and present refractory ascites as a TIPS

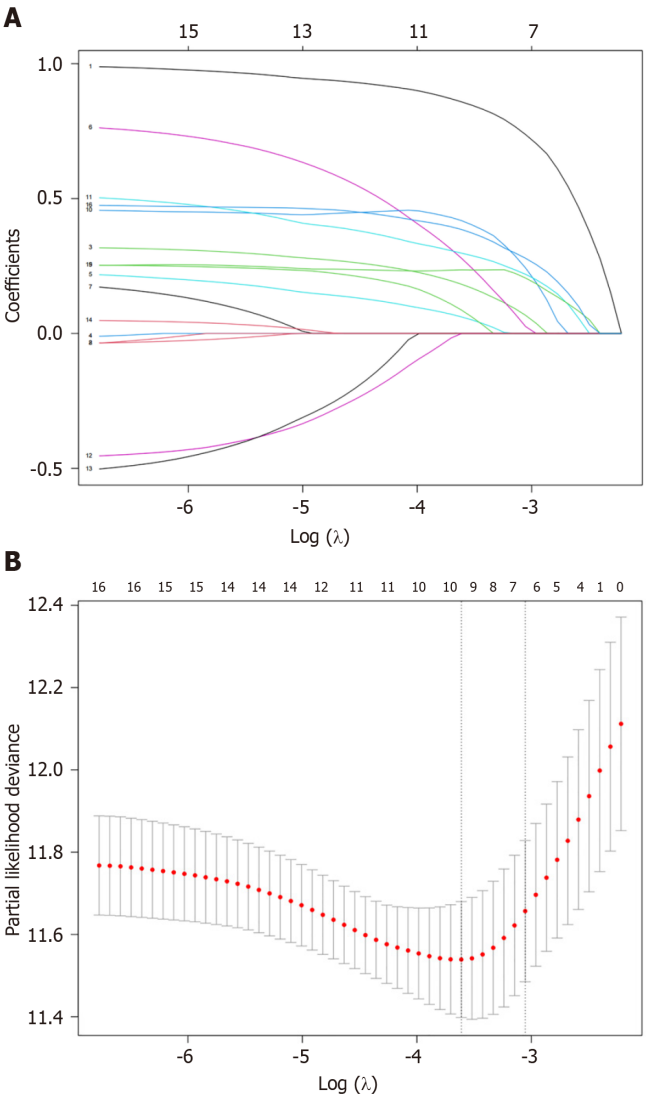


**Figure 2 Variable selection by least absolute shrinkage and selection operator regression.** A: Dynamic graph of variable selection *via* the least absolute shrinkage and selection operator regression; B: Selection of optimal predictive variables through cross-validation method.

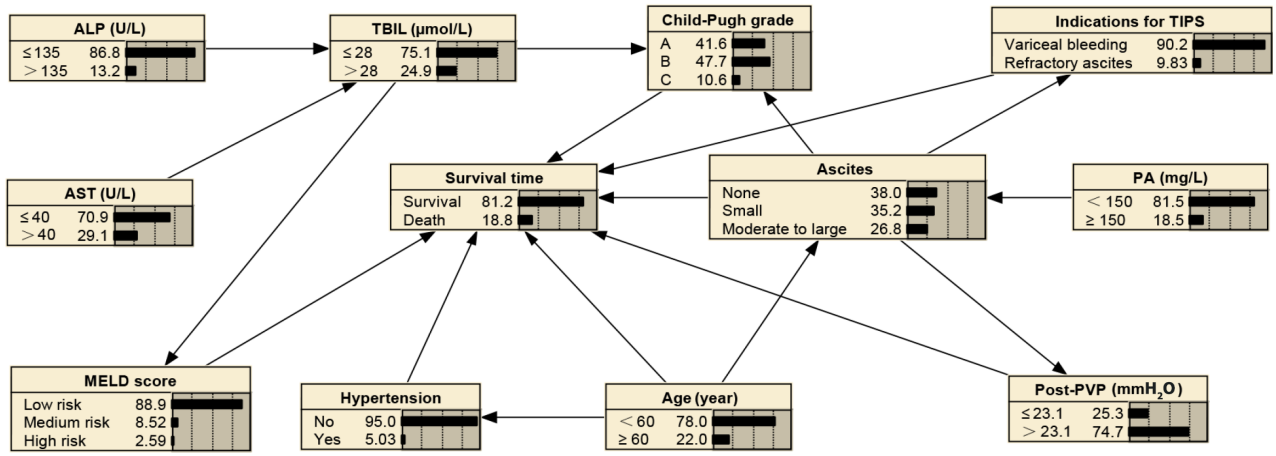


**Figure 3 Bayesian network with 12 nodes and 17 directed edges.** Nodes represent variables, and directed edges represent probabilistic dependence between connected nodes. The percentage in the figure represents the prior probability of each node. ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; MELD: Model for end-stage liver disease; TBIL: Total bilirubin; TIPS: Transjugular intrahepatic portosystemic shunt; PA: Prealbumin; PVP: Portal vein pressures.

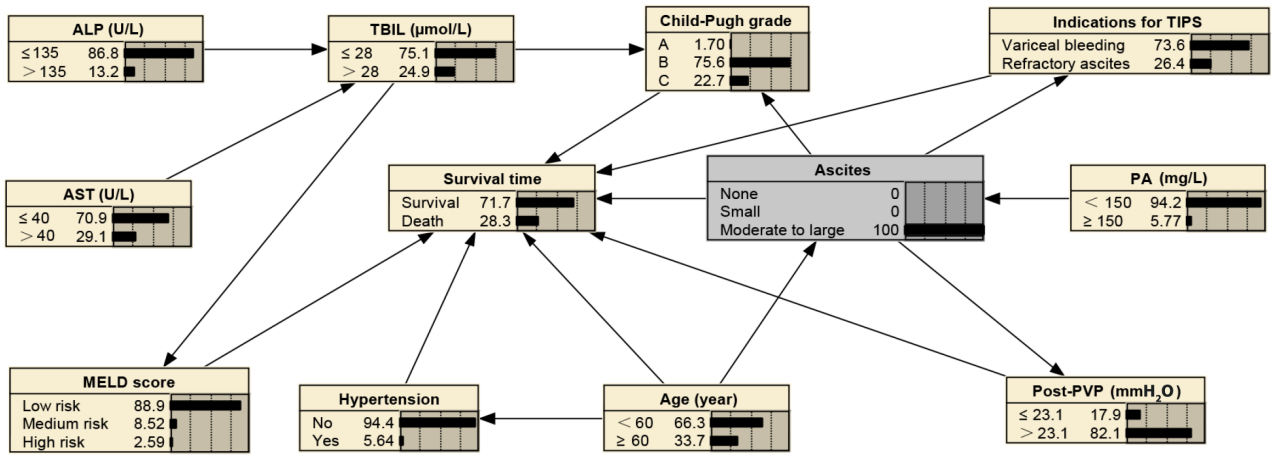


**Figure 4 Bayesian inference for patients with moderate-to-large ascites.** ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; MELD: Model for end-stage liver disease; TBIL: Total bilirubin; TIPS: Transjugular intrahepatic portosystemic shunt; PA: Prealbumin; PVP: Portal vein pressures.

indication, 82.50% of these patients are likely to experience moderate to severe ascites, 85.80% are likely to have PA levels below 150 mg/L, 80.10% are likely to have post-PVP exceeding 23.1 mm$H_2O$, and the 2-year mortality rate is predicted to increase to 49.60%, as shown in Figure 5. For patients aged 60 years or older with concurrent hypertension and TBIL levels exceeding 28 μmol/L, AST above 40 U/L, ALP greater than 135 U/L, and PA below 150 mg/L, there is a 76.40% probability of post-PVP exceeding 23.1 mm$H_2O$. In addition, these patients have a 44.20% likelihood of developing moderate-to-large ascites, a 36.50% chance of being classified as Child-Pugh grade C, an 8.42% probability of obtaining a high-risk MELD score, and a mortality rate that escalates to 52.40%, as detailed in Figure 6.

## DISCUSSION

PHT and its complications significantly impact the quality of life and prognosis of patients with cirrhosis[15]. Although TIPS is an essential therapeutic intervention that can effectively alleviate PHT-related symptoms, it can lead to postoperative complications that adversely affect patient survival. Consequently, developing an accurate prognostic prediction model is crucial for guiding clinical decision-making and enhancing patient management. Although studies like those by Huang *et al*[8], Sun *et al*[9], and Li *et al*[10] have focused on multiple prognostic factors, they have not comprehensively considered all relevant clinical variables and their interactions. For example, these studies did not thoroughly explore interrelations among factors like total TBIL, SCr, and sarcopenia and their collective impact on long-term patient survival. Conversely, our model is significantly more advantageous as it integrates complex prognostic factors and provides personalized predictions, thus capturing the multifaceted interactions of clinical variables. Our model not only covers a broader range of prognostic factors but also delves into their interactions and collective impact on long-term patient survival. In particular, our model reveals the dynamic changes and interdependencies among complex prognostic factors, an aspect not present in models like support vector machines, random forest algorithms, and nomograms. As an emerging modeling tool, BNs have shown significant potential in disease risk assessment and prognosis prediction[12]. This study marks the first application of BN methodology, which comprehensively considers various clinical variables and their relationships, provides more comprehensive and personalized prognostic information, and potentially improves treatment strategies and patient survival rates.

The study comprehensively assessed the biochemical markers of cirrhosis-induced PHT in patients, including liver function, renal function, coagulation status, and blood counts. It also considered various related variables, including ascites, PVP, concurrent diseases, and the etiology of cirrhosis. To select key determinants closely linked to post-TIPS survival, this study used a combination of Cox and LASSO regression methods, which facilitated the development of a model with improved explanatory power and enhanced predictive accuracy while concurrently minimizing the model's vulnerability to multicollinearity. The developed BN diagram illustrates the probabilistic dependencies among the post-TIPS survival time in patients with PHT and its predictive factors in a complex network. Various factors affect the post-TIPS survival time in patients with PHT *via* a network of complex interrelationships. This study's BN model revealed conditional dependencies among the variables of age, concurrent hypertension, ascites, indications for TIPS, post-PVP, the Child-Pugh grade, and the MELD score, which serve as parent nodes for the survival time and directly influence the overall survival of the patient. Age, functioning as a parent node, not only directly impacts the survival time but also indirectly influences it through its effect on the incidence of hypertension and ascites. The PA level, which is another parent node, is directly correlated with the ascites condition. Ascites is also a parent node, and it directly influences the survival time and additionally impacts it indirectly through the Child-Pugh grade, indications for TIPS, and post-PVP. AST and ALP, which are parent nodes for TBIL, directly influence its levels. TBIL is a parent node for the Child-Pugh grade and MELD score and indirectly impacts the survival time. An explanation of the reasons and mechanisms behind these interconnections is provided below.

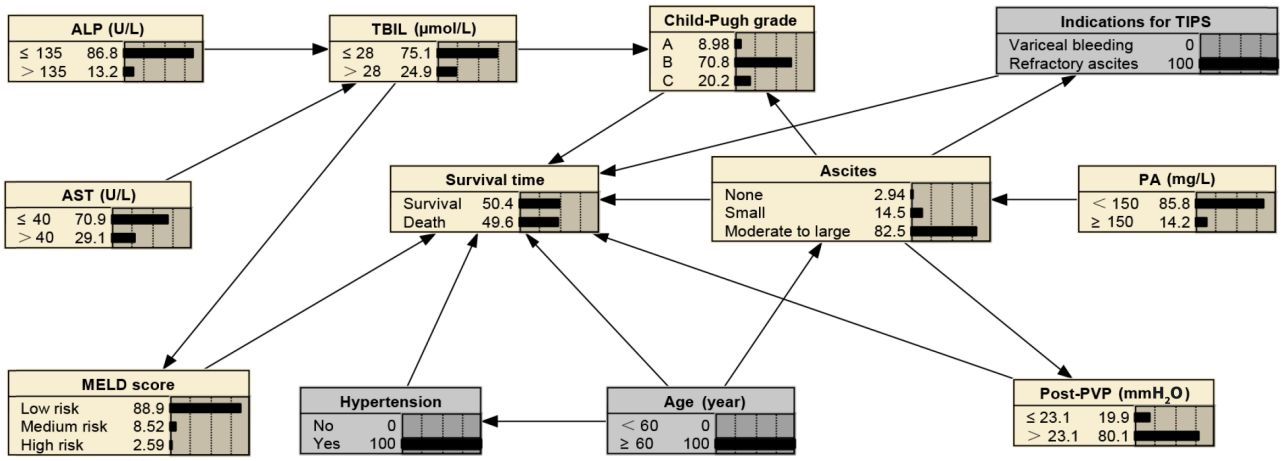


**Figure 5 Bayesian inference for patients aged ≥ 60 years with hypertension and refractory ascites as transjugular intrahepatic portosystemic shunt indications.** ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; MELD: Model for end-stage liver disease; TBIL: Total bilirubin; TIPS: Transjugular intrahepatic portosystemic shunt; PA: Prealbumin; PVP: Portal vein pressures.

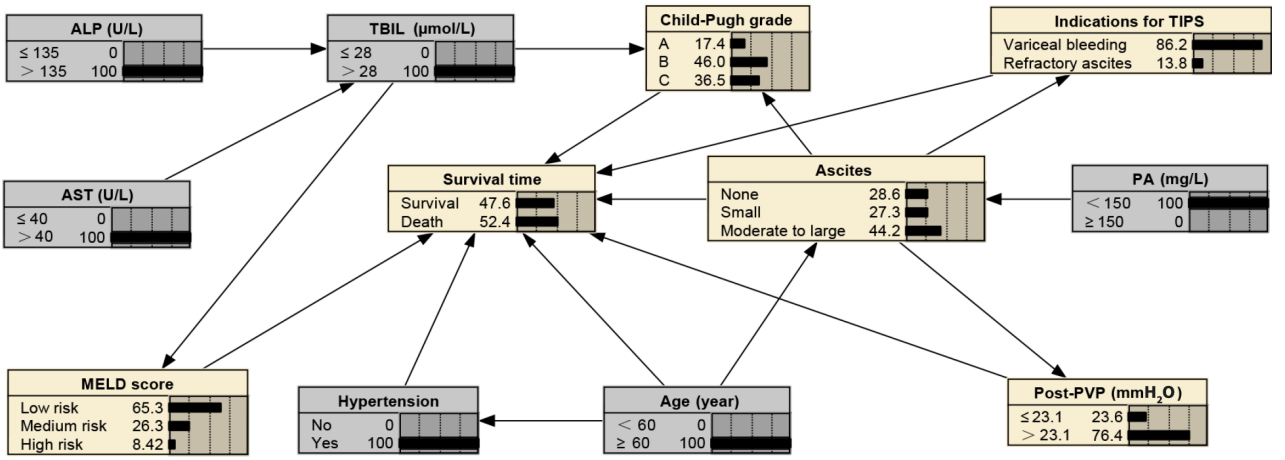


**Figure 6 Bayesian inference for patients aged ≥ 60 years with hypertension, alkaline phosphatase > 135 U/L, aspartate aminotransferase > 40 U/L, and prealbumin < 150 mg/L.** ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; MELD: Model for end-stage liver disease; TBIL: Total bilirubin; TIPS: Transjugular intrahepatic portosystemic shunt; PA: Prealbumin; PVP: Portal vein pressures.

With aging, patients often experience a decline in physiological functions and liver functional reserve, potentially leading to reduced surgical tolerance in older individuals and subsequently heightened surgical risks. Moreover, aging plays a critical role in the development of hypertension; the TIPS procedure modifies hepatic and portal vein hemodynamics, and the presence of hypertension can exacerbate cardiovascular strain, thus increasing postoperative risks and affecting patient survival prognosis[16]. In addition, aging may accompany alterations in other systems, such as drug metabolism and excretion, thus elevating the risk of ascites[17]. Ascites, often seen in advanced cirrhosis, is a marker of progression to a more severe liver disease stage and is closely associated with the survival time[18,19]. PA, which is synthesized by the liver and has a short half-life, is a sensitive indicator of both recent nutritional status and liver function [17]. Lower PA levels indicate reduced hepatic synthesis, suggesting a poor prognosis. When hepatic synthesis is impaired, the decrease in the level of PA may lead to a reduction in plasma colloid osmotic pressure, thus triggering or exacerbating ascites. Therefore, reduced PA levels play an important role in ascites development. In addition, ascites commonly occurs in patients with cirrhosis-induced PHT, with its severity closely linked to PVP. Although TIPS aims to reduce elevated PVP and alleviate ascites, the extent and severity of ascites indirectly affect post-PVP management. Increased post-PVP can increase the risk of postoperative complications like rebleeding, thus impacting patient survival prognosis[20,21]. Variceal bleeding and refractory ascites serve as primary indications for TIPS. Ascites influences the decision-making for TIPS, with refractory ascites indicating advanced-stage cirrhosis and poor prognosis[22]. AST and ALP, which are critical markers of liver function, signify liver cell damage or biliary obstruction when elevated[23,24], thus directly affecting bilirubin metabolism and leading to increased TBIL levels. TBIL levels, which reflect liver detoxification and excretion functions, play an important role in liver function assessments, such as the Child-Pugh grade and MELD score. Elevated TBIL levels imply diminished hepatic ability to process bilirubin, indicating advanced cirrhosis severity[25]. The Child-Pugh grade and MELD score are essential for assessing cirrhosis severity and prognosis[26,27]. These scores offer a comprehensive analysis of cirrhosis severity and survival prognosis by integrating various

parameters, including TBIL. Higher TBIL levels can lead to increased Child-Pugh grade and MELD score, signifying severe liver impairment and a worse prognosis, which could reduce the survival time. The study used a BN model to analyze factors affecting post-TIPS survival in patients with PHT; consequently, it uncovered various relationships, some of which are corroborated by existing research and others that require further validation. In the future, we will conduct prospective studies to more comprehensively validate these interrelationships.

In this study, we successfully developed and validated a BN-based model for accurately predicting post-TIPS survival outcomes in patients with PHT. The model demonstrated excellent performance, as evidenced by a BIC score of -3589.04, reflecting its high data fitting efficiency. Additionally, the model's robustness and reliability are corroborated by an average log-likelihood loss of 5.55 and a standard deviation of 0.16 from 10-fold cross-validation. Although the model demonstrates excellence in accuracy, precision, recall, and F1 score, an AUC value of 0.72 indicates potential for further enhancing its sensitivity and specificity in survival prognosis. Future efforts will aim to enhance the model's predictive power by expanding the training dataset, adjusting parameters, and optimizing the network structure. This study's focus on conditional probability inference highlights the BN's capacity to deduce unknown nodes from known ones, offering clinicians a powerful tool for precise patient health prediction and management. For example, the model can project risks of related health issues by analyzing existing patient clinical data, such as ascites severity and post-PVP changes. This accurate risk assessment enables more comprehensive evaluation of patient health by medical professionals, resulting in personalized treatment strategies. This approach is crucial for high-risk patients, allowing for proactive measures and strategic treatment adjustments to reduce specific complication risks. In addition, employing Bayesian inference strengthens the data-driven basis of clinical decision-making. Understanding these probabilistic dependencies empowers medical personnel to make more informed decisions during diagnosis and treatment. In particular, in predicting the post-TIPS survival time, factors like the Child-Pugh grade and MELD score may significantly influence patient survival under certain conditions. This understanding of conditional dependencies helps medical staff integrate long-term health forecasts into treatment plans and improves patient communication, thus enhancing their understanding and engagement.

This study has certain limitations. First, as a single-center retrospective study with a limited sample size, the stability and generalizability of its predictions need to be further validated by larger, multi-center prospective studies. Second, certain potential risk factors, such as portal vein diameter and sarcopenia, which were identified to impact the prognosis of patients having undergone TIPS surgery for PHT in other studies were not incorporated in this study. In addition, although the BN model's predictive accuracy is good enough to provide some useful insights, an AUC value of less than 0.8 suggests that there is room for improvement in its predictive capacity. Our future goal is to conduct multi-center studies with larger sample sizes, extended follow-up periods, and additional parameters to enhance the BN model for more accurate prediction of survival prognosis in patients having undergone TIPS surgery for PHT.

## CONCLUSION

In summary, this study signifies the first application of BN methodology in developing a survival prediction model for patients having undergone TIPS surgery for PHT. By incorporating numerous clinical variables, the model exposes the intricate conditional dependencies among these variables, showcasing its outstanding data fitting capabilities. It contributes to significantly enhancing the precision of survival prognosis for patients having undergone TIPS surgery for PHT while providing new analytical tools and strategies for patient evaluation, treatment planning, and disease management. Moreover, this study fills a critical gap in existing research by thoroughly exploring the interrelationships among various prognostic factors and their collective impact on patient outcomes, thus offering novel insights into the complex dynamics of PHT and its management after TIPS. Through this study, we aim to offer superior medical services to patients with PHT, ultimately improving their quality of life and survival rates. Finally, the application of BN in this study extends beyond mere survival outcome predictions, and it opens new perspectives and research directions for future clinical studies. Continuing this line of research will allow us to gain a deeper understanding of the risk factors associated with post-TIPS complications and their impact on patient outcomes, thus enabling the development of more scientific and effective treatment and management strategies for patients with PHT.

## ARTICLE HIGHLIGHTS

### *Research background*

Portal hypertension (PHT) secondary to cirrhosis leads to severe symptoms, exacerbating disease progression and adversely affecting survival rates. Transjugular intrahepatic portosystemic shunt (TIPS) is pivotal in managing PHT; however, its resultant complications can significantly impact patient prognosis. A thorough understanding of the interplay and mechanisms of various prognostic factors is crucial for enhancing treatment strategies and improving patient survival.

### *Research motivation*

There is a gap in existing research concerning the comprehensive exploration of the interrelationships and mechanisms of prognostic factors in PHT. We believe there is an urgent requirement for advanced modeling approaches to intricately analyze these interactions.

### *Research objectives*

To use Bayesian network (BN) methodology for extensive analysis of factors influencing the prognosis of PHT patients after TIPS. The objective involves elucidating the interdependencies among these factors and developing a BN model to predict patient survival after TIPS, thus facilitating informed clinical decisions.

### *Research methods*

In this study, we included 393 patients and used Cox and least absolute shrinkage and selection operator regression to select variables most relevant to prognosis, and we established a new BN survival prediction model for patients having undergone TIPS surgery for PHT.

### *Research results*

We successfully developed a BN-based survival prediction model with good predictive capabilities. Key factors impacting survival were identified, and the model showed high accuracy, precision, recall, and F1 score, with an AUC of 0.72, indicating its efficacy in survival prediction after TIPS in patients with PHT.

### *Research conclusions*

We developed a novel BN-based model for predicting survival in patients having undergone TIPS surgery for PHT. This model enhances the precision of survival prognosis and provides new analytical tools for patient evaluation, treatment planning, and disease management.

### *Research perspectives*

Data from other centers are essential for further validating the clinical usability of this novel model. Concurrently, continued research is imperative to deepen the understanding of post-TIPS complication risk factors and their impact on patient outcomes, thus guiding the development of more effective and scientific treatment strategies for patients with PHT.


## FOOTNOTES


**Author contributions:** Chen R designed the research, collected and organized the data, and wrote the initial draft of the manuscript; Zhang YZ guided the research design; Liu Z, Liu AL, and Zhang YW were involved in data collection and analysis; and Luo L managed the project and participated in the manuscript's review and editing; and all authors have read and approved the final version of the manuscript for publication.

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**Informed consent statement:** Informed consent was waived due to the retrospective cohort design of this study. For privacy reasons, patients' identifying information was replaced with codes before data extraction.

**Conflict-of-interest statement:** Authors declare no conflict of interest for this article.

**Data sharing statement:** The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.





**Country/Territory of origin:** China



**ORCID number:** Ling Luo 0009-0000-6855-7858.



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ORIGINAL ARTICLE

Retrospective Study

# Real-world efficacy and safety of tofacitinib treatment in Asian patients with ulcerative colitis

Kentaro Kojima, Kenji Watanabe, Mikio Kawai, Soichi Yagi, Koji Kaku, Maiko Ikenouchi, Toshiyuki Sato, Koji Kamikozuru, Yoko Yokoyama, Tetsuya Takagawa, Masahito Shimizu, Shinichiro Shinzaki

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**Kentaro Kojima, Kenji Watanabe, Mikio Kawai, Soichi Yagi, Koji Kaku, Maiko Ikenouchi, Toshiyuki Sato, Koji Kamikozuru, Yoko Yokoyama, Shinichiro Shinzaki,** Department of Gastroenterology, Hyogo Medical University, Nishinomiya 663-8501, Japan

**Kentaro Kojima, Masahito Shimizu,** Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

**Kenji Watanabe,** Department of Internal Medicine for Inflammatory Bowel Disease, The University of Toyama, Toyama 930-0194, Japan

**Tetsuya Takagawa,** Center for Clinical Research and Education, Hyogo Medical University, Nishinomiya 663-8501, Japan

**Corresponding author:** Kenji Watanabe, FACG, MD, PhD, Professor, Department of Gastroenterology, Hyogo Medical University, 1-1, Mukogawa-cho, Nishinomiya City, Hyogo Prefecture, Nishinomiya 663-8501, Japan. kenjiw@med.u-toyama.ac.jp

## Abstract

***BACKGROUND***

Real-world data on tofacitinib (TOF) covering a period of more than 1 year for a sufficient number of Asian patients with ulcerative colitis (UC) are scarce.

***AIM***

To investigate the long-term efficacy and safety of TOF treatment for UC, including clinical issues.

***METHODS***

We performed a retrospective single-center observational analysis of 111 UC patients administered TOF at Hyogo Medical University as a tertiary inflammatory bowel disease center. All consecutive UC patients who received TOF between May 2018 and February 2020 were enrolled. Patients were followed up until August 2020. The primary outcome was the clinical response rate at week 8. Secondary outcomes included clinical remission at week 8, cumulative persistence rate of TOF administration, colectomy-free survival, relapse after tapering of TOF and predictors of clinical response at week 8 and week 48.

***RESULTS***

The clinical response and remission rates were 66.3% and 50.5% at week 8, and 47.1% and 43.5% at week 48, respectively. The overall cumulative clinical remission rate was 61.7% at week 48 and history of anti-tumor necrosis factor-alpha (TNF-α) agents use had no influence ($P$ = 0.25). The cumulative TOF persistence rate at week 48 was significantly lower in patients without clinical remission than in those with remission at week 8 (30.9% *vs* 88.1%; $P$ < 0.001). Baseline partial Mayo Score was significantly lower in responders *vs* non-responders at week 8 (odds ratio: 0.61, 95% confidence interval: 0.45-0.82, $P$ = 0.001). Relapse occurred in 45.7% of patients after TOF tapering, and 85.7% of patients responded within 4 wk after re-increase. All 6 patients with herpes zoster (HZ) developed the infection after achieving remission by TOF.

*CONCLUSION*

TOF was more effective in UC patients with mild activity at baseline and its efficacy was not affected by previous treatment with anti-TNF-α agents. Most relapsed patients responded again after re-increase of TOF and nearly half relapsed after tapering off TOF. Special attention is needed for tapering and HZ.

**Key Words:** Ulcerative colitis; Tofacitinib; Janus kinase inhibitor; Real-world; Biologics



**Core Tip:** This is a retrospective single-center observational study to investigate the long-term efficacy and safety of tofacitinib (TOF) treatment for ulcerative colitis (UC), including clinical issues. TOF is more effective in low-activity UC patients and its efficacy is not affected by previous treatment with anti-tumor necrosis factor-alpha agents. Most patients in the clinical remission group at week 8 could continue TOF over a long follow-up period. Most relapsed patients responded again after re-increase of TOF and nearly half relapsed after tapering off TOF. Although most patients continue TOF without severe adverse events, careful monitoring for herpes zoster is necessary.

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## INTRODUCTION

Ulcerative colitis (UC) is an idiopathic disease causing chronic mucosal inflammation in the colorectum. The pathogenesis of UC is not fully established, but genetic factors and environmental factors are thought to be intricately intertwined with each other[1]. In addition, some factors may cause a dysregulated immune response in the intestinal tract that is involved in the onset and persistence of inflammation as a multifactorial disease[1]. Although various therapeutic agents have been developed, insufficient efficacy for remission induction and remission maintenance is observed. Several treatment options are currently available as advanced therapy, including calcineurin inhibitors, anti-tumor necrosis factor-alpha (TNF-α) agents (infliximab, adalimumab, golimumab), anti-α4β7 integrin antibodies (vedolizumab), and anti-interleukin-12/23 submit p40 antibodies (ustekinumab) for patients with intractable UC. In 2018, the small molecule Janus kinase (JAK) inhibitor tofacitinib (TOF) was approved for use in Japan. The selective JAK1 inhibitors filgotinib and upadacitinib were also recently approved in Japan.

Cytokines are involved in both intestinal homeostasis and pathologic processes associated with inflammatory bowel disease (IBD)[2]. TOF prevents the activation of JAK, phosphorylation of signal transducer and activator of transcription (STAT), and translocation to the nucleus to activate gene transcription, and thus downregulates cytokine production[3]. The JAK/STAT pathway plays an important role in cell growth and survival, differentiation, and proliferation. JAK consists of JAK1, JAK2, JAK3, and tyrosine kinase 2, and TOF is a non-selective pan-JAK inhibitor, targeting mainly JAK1/JAK3[4,5]. TOF is orally bioavailable and has predictable pharmacokinetics and no immunogenicity in contrast to monoclonal antibody therapies such as the anti-TNF-α agents used for the treatment of IBD[2].

The efficacy and safety of TOF for moderately to severely active UC were globally shown in a phase II trial; and 2 phase III induction studies (OCTAVE Induction 1 and 2); a phase III maintenance study (OCTAVE Sustain); and an open-label, long-term extension study (OCTAVE Open)[6-8]. In Japanese patients, post hoc analyses of TOF treatment in 2 studies (OCTAVE Induction 1 and OCTAVE Sustain) are reported[9]. The importance of real-world data, however, has become increasingly recognized in recent years because the setting of a clinical (drug) trial is a special situation[10]. Additionally, genetic backgrounds and phenotypes of IBD differ considerably between Asian and Western patients[11, 12]; thus, it is critical to determine the appropriate treatment targets, outcomes, and responses in populations with different genetic backgrounds[13]. It is well-known that the risk of herpes zoster (HZ) as an adverse event associated with TOF is greater in Asian UC patients than in Western UC patients[14]. The United States Food and Drug Administration noted the risk of major adverse cardiovascular events, malignancy, and thrombosis for JAK inhibitors in patients with

other immune-mediated diseases, *e.g.*, rheumatoid arthritis[15]. It is possible, however, that the difference in the risks depends on the disease or geographical region.

Only 1 published study of more than 100 Asian UC cases with a 1-year observation period focusing on the efficacy and safety of TOF is available from Korea[16]. Real-world data, however, including clinical issues of TOF, such as relapse rate after tapering TOF or efficacy of re-increasing the dosage of TOF, with a sufficient number of subjects are essential for optimizing treatment with TOF and comparing with the other data of JAK1 inhibitors to optimize the JAK inhibitor treatment strategies in Asian UC patients. The aim of this investigation was to evaluate the clinical efficacy, safety, and clinical issues related to TOF in more than 100 Japanese UC patients with a median observation period of more than 1 year in our specialized IBD center.

## MATERIALS AND METHODS

### Study design and patients

We performed a retrospective single-center observational analysis to investigate the efficacy and safety of TOF for intractable UC patients at the Hyogo Medical University as a tertiary IBD center. This investigation was approved by the Institutional Ethics Committee at our institute (number 3030).

All consecutive UC patients who received TOF between May 2018 and February 2020 were enrolled. Patients were followed up until August 2020. The diagnosis of UC was based on the diagnostic criteria of the Research Committee on Inflammatory Bowel Disease in Japan[17]. For evaluation of the efficacy, we excluded patients with IBD-unclassified, patients who underwent total colectomy, and patients who could not be followed up ≥ 8 wk after the initiation of TOF. Patients with a partial Mayo Score (pMS) ≤ 2 at TOF initiation were also excluded[18]. For the safety evaluation, all patients who were administered TOF were included. Patients aged ≤ 16 years were excluded from assessment of the efficacy and safety of TOF.

### Data collection

All data on patient demographics were retrieved from electronic medical records. Data were collected on age, sex, duration of UC, disease extent according to the Montreal classification[19], family history of IBD, smoking status, comorbidities, past history of HZ, disease activity according to the total or pMS[18], endoscopic activity according to the Mayo endoscopic subscore, intractability to steroids, details of previous and concomitant UC therapies, and details of TOF treatment (dosage and duration). Laboratory data included white blood cell count, lymphocyte count, hemoglobin, serum albumin, C-reactive protein (CRP), and total cholesterol levels. Potential adverse events related to TOF were recorded in all patients who received at least 1 dose of TOF.

### Outcomes and definitions

All patients were administered TOF (10 mg p.o.) twice daily as induction therapy. After achieving clinical remission, the dosage of TOF was tapered and then withdrawn according to the patient's condition. If relapse occurred after tapering or withdrawing, dose escalation or re-administration were accepted.

The primary outcome was the proportion of patients with a clinical response (defined as a decrease in pMS ≥ 2 points from baseline with an accompanying decrease in the rectal bleeding subscore ≥ 1 point or an absolute rectal bleeding subscore ≤ 1 point). Secondary outcomes included clinical remission (defined as pMS ≤ 2, with no subscore > 1 and a rectal bleeding subscore of 0) at week 8[20], steroid-free remission (defined as clinical remission without corticosteroid use at that time point), cumulative remission rate among patients who achieved clinical remission at week 8, cumulative persistence rate of TOF administration, colectomy-free survival, relapse after tapering of TOF (defined as increasing dosage of TOF or concomitant treatment drug, addition of treatment drug, or an increase in a pMS of at least 2 points or an increase with absolute rectal bleeding subscore ≥ 1 point from the time of TOF reduction), and predictors of clinical response at week 8 and week 48. A delayed responder was defined as a patient who did not achieve clinical remission at week 8 but had achieved clinical remission by week 24. Complete endoscopic remission was defined as a Mayo endoscopic score (MES) of 0. Endoscopic remission was defined as a MES ≤ 1 and endoscopic response was defined as a decrease from baseline in the MES of at least 1 point. Endoscopic efficacy at week 16 was assessed in 25 patients who underwent endoscopy by week 16, and the other 14 clinical non-responders who did not undergo endoscopy until week 16 were regarded as endoscopic non-responders with MES ≥ 2 at week 16. Patients who did not undergo colonoscopy were excluded from the evaluation of endoscopic efficacy despite being in clinical remission. Treatment failure was defined as TOF discontinuation due to symptomatic recurrence or adverse events, or additional therapy. Patients considered to have failed treatment were counted as non-responders. For example, a patient who discontinued TOF at week 8 was classified as a non-responder until week 60. If TOF was discontinued due to the desire to have children or the patient was transferred, it was censored at that time. The follow-up period was defined as the time between the initiation of TOF and the last visit or discontinuation of TOF until August 2020.

### Statistical analyses

In the descriptive statistics of quantitative variables, the median and interquartile range (IQR) were calculated depending on whether the data were normally distributed. Categorical variables are presented as percentage with 95% confidence intervals (CI). We used the Mann–Whitney U test to compare the quantitative variables and Fisher's exact test to compare categorical variables for patients in the responder and non-responder groups at week 8 and week 48. Paired *t* test was

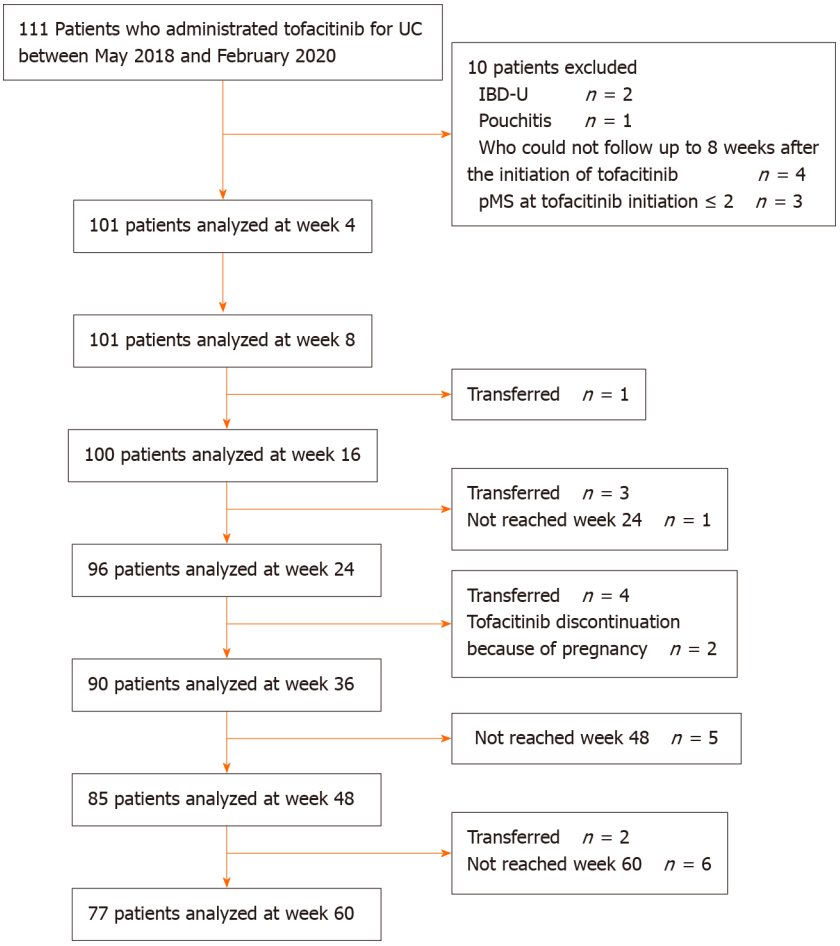


**Figure 1 Flow diagram of the participants analyzed.** IBD-U: Inflammatory bowel disease-unclassified; pMS: Partial Mayo Score; UC: Ulcerative colitis.

used to determine the level of significance of change in total cholesterol levels over time during the treatment of TOF. Survival was analyzed using the Kaplan-Meier method and log-rank test. *P* values for log-rank trend tests were examined whether increased score of pMS at baseline stratified by number of previous anti-TNF-α agent failures associate with the cumulative remission rate or persistence rate of TOF administration. Variables associated with predictors of the baseline characteristics of the efficacy at week 8 were explored using a logistic regression model. *P* values < 0.05 were considered as statistically significant. Statistical analyses were performed with EZR ver. 1.38 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)[21].

## RESULTS

### *Patient's characteristics at baseline*

The study included 111 patients treated with TOF between May 2018 and February 2020. Among them, 101 patients were included in the assessment of the efficacy of TOF after excluding 10 patients (Figure 1). The median follow-up time was 365 (IQR: 231–500) d. Patients' baseline characteristics are shown in Table 1. The median age was 35.0 (IQR: 28.0–47.0) years, 60 (59.4%) patients were male, and the median disease duration was 4.8 (IQR: 1.5–10.0) years. Most patients had pancolitis-type UC (82.2%). Seven (6.9%) patients had a family history of IBD and 14 (13.9%) were current smokers. Seven (6.9%) patients had hypertension, 6 (5.9%) had dyslipidemia, 3 (3.0%) had diabetes, and 2 (2.0%) had thrombosis. Only 1 (1.0%) patient had a past history of HZ. Forty-eight (47.5%) patients were steroid-dependent and 51 (50.5%) were steroid-refractory, and 23 (22.8%) received systemic corticosteroid therapy at TOF administration. Seventy (69.3%) patients had previously received an immunomodulator, 27 (26.7%) received cytapheresis, 31 (30.7%) received tacrolimus, and 67 (66.3%) received biologics. Sixty-three (62.4%) patients received anti-TNF-α agents, 11 (10.9%) received vedolizumab, and 7 (6.9%) received both. The number of anti-TNF-α agent failures was 44 (43.6%) patients with 1 failure, 17 (16.8%) patients with 2 failures, and 2 (2.0%) patients with 3 failures. The median pMS was 6.0 (IQR: 4.0-7.0) and median CRP was 0.3 (IQR: 0.1-1.0) mg/dL. At baseline, 54 (53.5%) patients underwent endoscopy, with a median MES of 2.0 (IQR: 2.0-3.0) and a median total Mayo Score of 9.0 (IQR: 7.0-10.0).

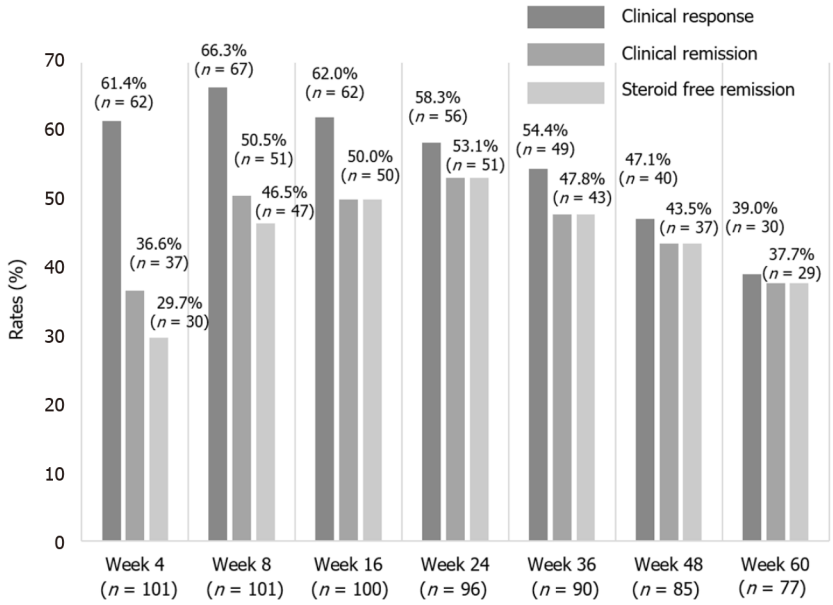


**Figure 2 Rate of clinical response, clinical remission, and steroid-free remission at weeks 4, 8, 16, 24, 36, 48, and 60.**

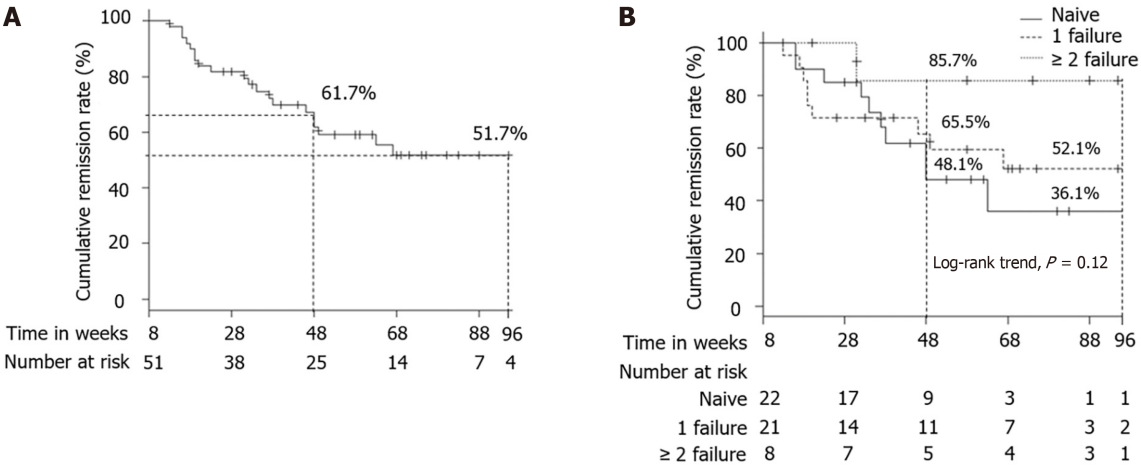


**Figure 3 Cumulative remission rate.** A: Cumulative remission rate in patients who achieved clinical remission at week 8; B: Cumulative remission rates according to previous history of anti-tumor necrosis factor α agent failure (naive *vs* 1 failure *vs* ≥ 2 failure) in patients who achieved clinical remission at week 8.

### *Clinical efficacy of TOF*

The proportion of patients with a clinical response, clinical remission, and steroid-free remission was 61.4% (62/101), 36.6% (37/101), 29.7% (30/101), respectively, at week 4, and 66.3% (67/101), 50.5% (51/101), 46.5% (47/101), respectively, at week 8 (Figure 2). After week 16, all patients with clinical remission achieved steroid-free remission. Seven (6.9%) patients were delayed responders. The proportion of patients with clinical response and (steroid-free) clinical remission was 47.1% (40/85) and 43.5% (37/85), respectively, at week 48, and 39.0% (30/77) and 37.7% (29/77), respectively, at week 60.

The cumulative remission rate in patients who achieved clinical remission at week 8 was 61.7% at week 48 and 51.7% at week 96 (Figure 3A). According to a previous history of anti-TNF-α agent failure (naive *vs* 1 failure *vs* ≥ 2 failure), the cumulative remission rate was 48.1% *vs* 65.5% *vs* 85.7% at week 48, and 36.1% *vs* 52.1% *vs* 85.7% at week 96 (Figure 3B). The cumulative remission rate did not differ significantly among the 3 groups (log-rank trend test, $P = 0.12$).

On the other hand, the cumulative persistence rate of TOF administration was 78.2% at week 8, 67.9% at week 24, 60.2% at week 48, and 57.2% at week 96 (Figure 4A). According to the raw data of pMS at baseline, we divided participants into 3 groups (Group 1: pMS 3-4, Group 2: pMS 5-6, Group 3: pMS 7-9), and the cumulative persistence rate of TOF administration among 3 groups, respectively, was 96.3%, 80.0%, and 64.1% at week 8; 80.0%, 67.2%, and 39.8% at week 48; and 80.0%, 67.2%, and 31.8% at week 96 (log-rank trend test, $P < 0.001$) (Figure 4B). Additionally, the cumulative persistence rate of TOF administration between patients who were in clinical remission and non-clinical remission at week 8 was, respectively, 98.0% and 36.8% at week 24, 88.1% and 30.9% at week 48, 82.9% and 30.9% at week 96 ($P < 0.001$; Figure 4C).

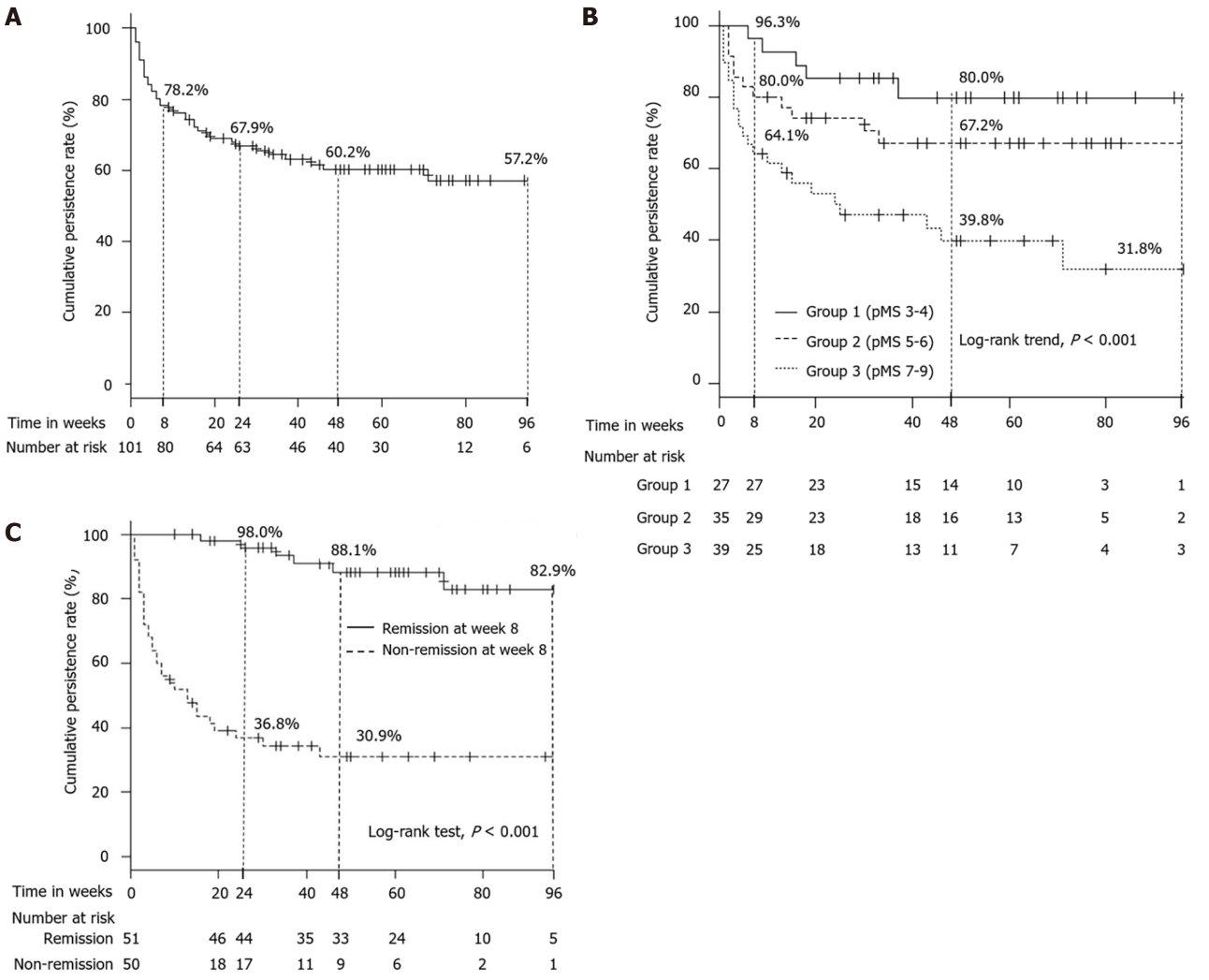


**Figure 4 Cumulative persistence rate of tofacitinib administration.** A: Overall cumulative persistence rate of tofacitinib administration; B: Cumulative persistence rate of tofacitinib administration according to partial Mayo Score (pMS) at baseline (Group 1: pMS 3-4, Group 2: pMS 5–6, Group 3: pMS 7–9); C: Cumulative persistence rate of tofacitinib administration between patients who were classified as in clinical remission or non-clinical remission at week 8. pMS: Partial Mayo Score.

Nine (8.9%) patients required a colectomy during the follow-up period; 2 due to primary failure of TOF and 7 due to failure of remission maintenance therapy after withdrawing the TOF. After excluding cases that transferred, could not be followed up, or whose treatment was discontinued due to pregnancy (Figure 1), the colectomy-free survival rate was 91.9% at week 48 and 89.1% at week 96 (Figure 5).

For endoscopic efficacy at week 16, the complete endoscopic remission rate was 7.7% (3/39), the endoscopic remission rate was 23.1% (9/39), and the endoscopic response rate was 38.5% (15/39).

### *Baseline factors associated with the efficacy of TOF*

Clinical and demographic baseline characteristics between responders and non-responders at week 8 are shown in Table 2. Baseline pMS of responders at week 8 was significantly lower than that of non-responders [5.0 (IQR: 4.0-7.0) *vs* 7.0 (IQR: 5.3-8.0), $P < 0.001$]. In multivariate analysis, lower baseline pMS was independently associated with responders at week 8 [Odds ratio (OR): 0.61, 95%CI: 0.45-0.82, $P = 0.001$].

### *Relapse rate after tapering and efficacy of re-increasing the dosage of TOF*

Figure 6 shows the number of patients treated with 5 mg or 10 mg twice daily at each time point in whole patients. Seventy-one (70.3%) patients were administered TOF at week 8. Among of them, 36 (35.6%) underwent tapering of TOF (5 mg twice daily) after achieving clinical remission, and the median period from baseline to the beginning of tapering was 92 (IQR: 63–120) d. The relapse rate after tapering (excluding 1 patient that withdrew from TOF due to an adverse event) was 45.7% (16/35) (Figure 7A). In these 16 patients with relapse, 14 re-increased the dosage of TOF. The clinical response and remission rate of re-increasing the dosage was, respectively, 85.7% (12/14) and 57.1% (8/14) at week 4, 84.6 % (11/13) and 69.2 % (9/13) at week 12 (Figure 7B). The median period from beginning of tapering to re-increasing the dosage was 95 (IQR: 38–216) d.

**Table 1 Patient characteristics at baseline**

| Characteristic | *n* = 101 |
|---|---|
| Age, years, median (IQR) | 35 (28.0, 47.0) |
| Sex | |
| Male, *n* (%) | 60 (59.4) |
| Female, *n* (%) | 41 (40.6) |
| Duration of UC, years, median (IQR) | 4.8 (1.5, 10.0) |
| Disease extent, *n* (%) | |
| Left-sided colitis | 18 (17.8) |
| Pancolitis | 83 (82.2) |
| Family history of IBD, *n* (%) | 7 (6.9) |
| Smoking classification, *n* (%) | |
| Never smoked | 66 (65.3) |
| Current smoker | 14 (13.9) |
| Ex-smoker | 21 (20.8) |
| Comorbidities, *n* (%) | |
| Hypertension | 7 (6.9) |
| Dyslipidemia | 6 (5.9) |
| Diabetes mellitus | 3 (3.0) |
| Thrombosis | 2 (2.0) |
| Past history of herpes zoster, *n* (%) | 1 (1.0) |
| Total Mayo score (*n* = 54), median (IQR) | 9.0 (7.0,10.0) |
| Partial Mayo score, median (IQR) | 6.0 (4.0, 7.0) |
| Mayo endoscopic subscore (*n* = 54), median (IQR) | 2.0 (2.0, 3.0) |
| 1, *n* (%) | 3.0 (5.0, 6.0) |
| 2, *n* (%) | 26 (48.1) |
| 3, *n* (%) | 25 (46.3) |
| Intractability, *n* (%) | |
| Steroid dependent | 48 (47.5) |
| Steroid refractory | 51 (50.5) |
| C-reactive protein (mg/dL), median (IQR) | 0.3 (0.1, 1.0) |
| Albumin (g/dL), median (IQR) | 3.9 (3.5, 4.2) |
| White blood cells (/μL), median (IQR) | 7180.0 (5490.0, 9160.0) |
| Lymphocytes (/μL), median (IQR) | 1506.0 (1083.0, 1874.0) |
| Hemoglobin (g/dL), median (IQR) | 12.3 (11.1, 13.8) |
| Total Cholesterol (mg/dL) (*n* = 71), median (IQR) | 179.0 (163.0, 199.0) |
| Concomitant drugs at baseline, *n* (%) | |
| 5-aminosalicylic acid | 68 (67.3) |
| Systemic corticosteroid | 23 (22.8) |
| Topical corticosteroid | 22 (21.8) |
| History of treatment at baseline | |
| Previous corticosteroid, *n* (%) | 99 (98.0) |
| Previous immunomodulator, *n* (%) | 70 (69.3) |

| | |
|---|---|
| Previous cytapheresis, *n* (%) | 27 (26.7) |
| Previous tacrolimus, *n* (%) | 31 (30.7) |
| Previous biologics, *n* (%) | 67 (66.3) |
| Anti-TNF-α agent, *n* (%) | 63 (62.4) |
| Infliximab | 38 (37.6) |
| Adalimumab | 27 (26.7) |
| Golimumab | 19 (18.8) |
| Number of anti-TNF-α agent failure, *n* (%) | |
| 1 agent | 44 (43.6) |
| 2 agents | 17 (16.8) |
| 3 agents | 2 (2.0) |
| Vedolizumab, *n* (%) | 11 (10.9) |
| Anti-TNF-α agent and vedolizumab, *n* (%) | 7 (6.9) |

IBD: Inflammatory bowel disease; IQR: Interquartile range; TNF-α: Tumor necrosis factor α; UC: Ulcerative colitis.

**Table 2 Baseline factors associated with the efficacy of Tofacitinib at week 8**

| Variable | Univariate analyses | | | Multivariate analyses | | |
|---|---|---|---|---|---|---|
| | Non-responder (*n* = 34) | Responder (*n* = 67) | *P* value | Odds ratio | 95%CI | *P* value |
| Age, years, median (IQR) | 32.5 (27.5, 46.0) | 37.0 (28.0, 47.5) | 0.27 | 1.02 | 0.99-1.05 | 0.28 |
| Sex (M/F), *n* | 20/14 | 40/27 | 1.00 | | | |
| Baseline partial Mayo score, median (IQR) | 7.0 (5.3, 8.0) | 5.0 (4.0, 7.0) | < 0.001 | 0.61 | 0.45-0.82 | 0.001 |
| Disease duration, years, median (IQR) | 6.2 (1.7, 9.9) | 4.5 (1.6, 10.0) | 0.84 | | | |
| Disease extent, *n* (%) | | | | | | |
| Left-sided colitis | 8 (23.5) | 10 (14.9) | | | | |
| Extensive colitis | 26 (76.5) | 57 (85.1) | 0.29 | | | |
| Family history of IBD, *n* (%) | 3 (8.8) | 4 (6.0) | 0.69 | | | |
| Current smoker, *n* (%) | 5 (14.7) | 9 (13.4) | 1.00 | | | |
| Intractability (dependent/refractory), *n* | 16/18 | 32/33 | 1.00 | | | |
| Albumin, g/dL, median (IQR) | 3.9 (3.5, 4.3) | 3.9 (3.6, 4.2) | 0.88 | | | |
| C-reactive protein, mg/dL, median (IQR) | 0.2 (0.1, 0.7) | 0.3 (0.1, 1.1) | 0.49 | | | |
| White blood cells, /μL, median (IQR) | 6660.0 (5107.5, 8415.0) | 7260.0 (5760.0, 9460.0) | 0.15 | 1.00 | 1.00-1.00 | 0.17 |
| Hemoglobin, g/dL, median (IQR) | 12.4 (11.1, 13.7) | 12.3 (11.2, 14.0) | 1.00 | | | |
| Baseline systemic corticosteroid use, *n* (%) | 8 (23.5) | 15 (22.4) | 1.00 | | | |
| Previous immunomodulator, *n* (%) | 24 (70.6) | 46 (68.7) | 1.00 | | | |
| Previous tacrolimus, *n* (%) | 11 (32.4) | 20 (29.9) | 0.82 | | | |
| Previous biologics, *n* (%) | | | | | | |
| Previous anti TNF-α agent | 22 (64.7) | 41 (61.2) | 0.83 | | | |
| Previous vedolizumab | 4 (11.8) | 7 (10.4) | 1.00 | | | |

CI: Confidence interval; IBD: Inflammatory bowel disease; IQR: Interquartile range; TNF-α: Tumor necrosis factor α.

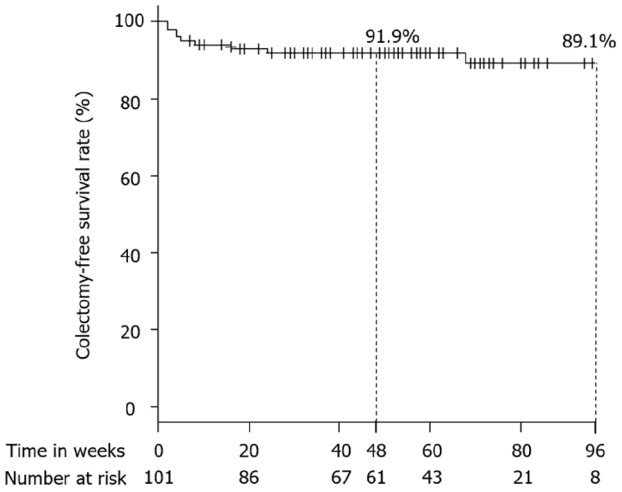


**Figure 5 Colectomy-free survival after the initiation of tofacitinib.**

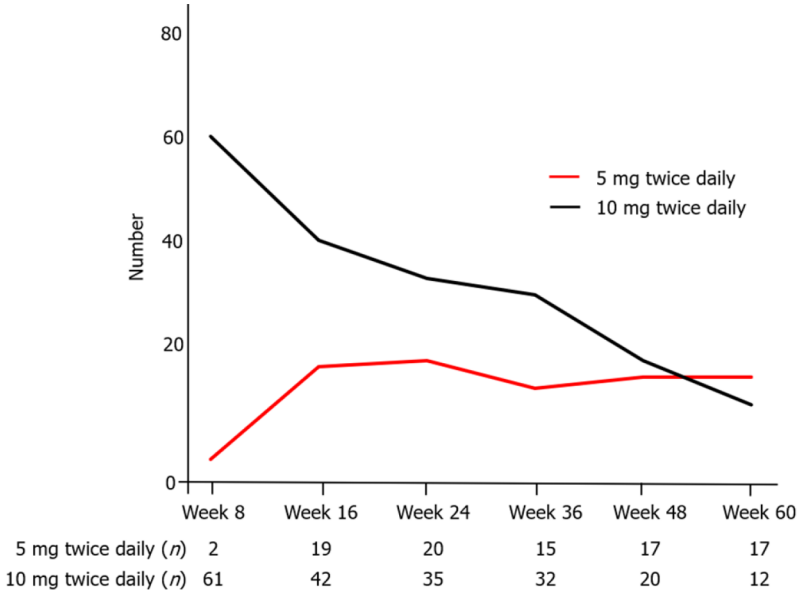


**Figure 6 Number of patients in each dosage group of tofacitinib administration at each time point.**

### *Safety profile*

All 111 patients who received TOF were included in the safety evaluation and observed for 80.5 patient-years. A total of 58 adverse events occurred during the follow-up period, 38 (34.2%) patients experienced at least 1 adverse event. All adverse events are shown in Table 3. Seven (6.3%) patients discontinued TOF due to adverse events; 3 (2.7%) with fever, and 1 each (0.9%) with bronchitis, elevation of creatine kinase, lower leg edema, general fatigue, or sore throat.

The most common adverse event was hypercholesterolemia in 17 (15.3%) patients, and all 17 patients were administered an oral antihyperlipidemic drug. Total cholesterol levels were not significantly different at weeks 0, 4, and 8 between responders and non-responders at week 8 (Figure 8).

In terms of infectious disease, HZ developed in 6 patients, upper respiratory tract infection in 5, influenza in 3, herpes labialis in 1, norovirus enteritis in 1, and *Clostridioides difficile* colitis in 1. Among patients with HZ, the median age was 51 (IQR: 35–54) years, and the median lymphocyte count was 1466 (IQR: 1167–1750) (/μL) (Table 4). There were no specific features for TOF dosage, duration of TOF administration, or concomitant drug in these patients. All patients with HZ developed it while in the clinical remission stage. The median age [50 (IQR: 34–54) *vs* 35 (IQR: 28–47) years, $P = 0.20$] and lymphocyte count [1369 (IQR: 1131–1504) *vs* 1485 (IQR: 1064–1892) (/μL), $P = 0.45$] were also not different in patients with HZ and without HZ (Figure 9). All patients that had HZ were treated with oral medications. Three patients continued TOF administration, 2 patients were withdrawn temporarily, and 1 was discontinued permanently. HZ occurred in multiple dermatomes in only 1 patient, and postherpetic neuralgia remained in 1 patient.

Deep vein thrombosis was observed in 1 patient; a 53-year-old man with emphysema developed deep vein thrombosis in the left soleus vein with an unknown trigger. He was examined with ultrasonography because his D-dimer was slightly elevated (from 0.6 μg/mL to 2.4 μg/mL). The size of the thrombus was unchanged throughout the observation

**Table 3 Adverse event, *n* (%)**

| Safety profile | | Incidence rates per 100 patient-years |
|---|---|---|
| Hypercholesterolemia | 17 (15.3) | 21.1 |
| Herpes zoster | 6 (5.4) | 7.5 |
| Upper respiratory tract infection | 5 (4.5) | 6.2 |
| Fever | 4 (3.6) | 5.0 |
| Headache | 3 (2.7) | 3.7 |
| Influenza | 3 (2.7) | 3.7 |
| Lower leg edema | 2 (1.8) | 2.5 |
| General fatigue | 2 (1.8) | 2.5 |
| Sore throat | 2 (1.8) | 2.5 |
| Bronchitis | 1 (0.9) | 1.2 |
| Herpes labialis | 1 (0.9) | 1.2 |
| Norovirus enteritis | 1 (0.9) | 1.2 |
| Clostridioides difficile colitis | 1 (0.9) | 1.2 |
| Folliculitis | 1 (0.9) | 1.2 |
| Palpitations | 1 (0.9) | 1.2 |
| Joint pain | 1 (0.9) | 1.2 |
| General pain | 1 (0.9) | 1.2 |
| Dizziness | 1 (0.9) | 1.2 |
| Sleepiness | 1 (0.9) | 1.2 |
| Deep vein thrombosis | 1 (0.9) | 1.2 |
| Dysplasia | 1 (0.9) | 1.2 |
| Elevation of creatine kinase | 1 (0.9) | 1.2 |
| Abnormal liver function tests | 1 (0.9) | 1.2 |

period even while continuing TOF without administering an antithrombotic agent.

## DISCUSSION

The main purpose of our study was to evaluate the efficacy and safety of TOF in the real-world. Our study provides support for the efficacy of both short-term and long-term TOF treatment in active UC patients. Two-thirds of the patients had a clinical response and half achieved clinical remission at week 8. In addition, 44% of patients achieved corticosteroid-free remission at week 48 and the cumulative remission rate at week 48 among those patients in clinical remission at week 8 was 62%.

These results are fairly consistent with previously reported real-world studies. With regard to short-term efficacy, Honap *et al*[22] reported that a clinical response [defined as the reduction of The Simple Clinical Colitis Activity Index (SCCAI) or pMS ≥ 3] was achieved in 74% at week 8 and clinical remission (SCCAI ≤ 2 or pMS ≤ 1) was achieved in 57% of patients in a multicenter retrospective study involving 134 UC patients (83% previously treated with biologics). Similarly, Chaparro *et al*[23] reported that a clinical response (reduction in pMS ≥ 3 points and at least 30% from baseline, with a decrease ≥ 1 point in the rectal bleeding subscale) was achieved in 60% at week 8 and clinical remission (pMS < 2) was achieved in 31% of patients in a multicenter prospective study involving 113 UC patients (100% previously treated with biologics). With regard to long-term efficacy, Lair-Mehiri *et al*[24] reported steroid-free clinical remission (pMS < 3 with a combined stool frequency and rectal bleeding subscore ≤ 1) in 34% of patients at week 48 in a multicenter retrospective study involving 38 UC patients (100% previously treated with biologics). Chaparro *et al*[23] reported that 38% of patients who achieved remission at week 8 relapsed over time (median of exposure to TOF, 44 wk).

In the OCTAVE Induction 1 and 2 trials (almost 50% previously treated with anti-TNF-α agent), 19% and 17% of UC patients treated with TOF achieved remission at week 8, and 60% and 55% achieved a clinical response at week 8, respectively. In the OCTAVE Sustain trial, which included patients who had a clinical response to induction therapy, the sustained remission rate at week 52 was 37% in the 5 mg TOF twice-daily group and 47% in the 10 mg TOF twice-daily

**Table 4 Characteristics of patients who developed herpes zoster**

| Case | Age | Sex | Prior TNF-α failure | TOF dose (mg/day) | Week of onset | pMS at onset | Lymphocyte count (/μL) | Concomitant drug | Therapy for HZ | TOF therapy | Area of HZ | Postherpetic neuralgia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 68 | F | (+) | 10 | 30 | 1 | 1362 | 5ASA; PPI; ARB | OAD | Continuation | Lower leg | (+) |
| 2 | 30 | F | (+) | 20 | 5 | 0 | 1570 | Prednisolone 10 mg | OAD | Resumption after drug withdrawal | Head; Neck; Back | (-) |
| 3 | 25 | F | (+) | 10 | 23 | 1 | 1102 | 5ASA | OAD | Continuation | Back | (-) |
| 4 | 48 | M | (+) | 10 | 31 | 1 | 1810 | 5ASA | OAD | Resumption after drug withdrawal | Abdomen | (-) |
| 5 | 54 | F | (-) | 20 | 5 | 1 | 2127 | Iron preparations | OAD | Discontinuation | Buttocks | (-) |
| 6 | 53 | M | (+) | 10 | 26 | 0 | 922 | (-) | OAD | Continuation | Back | (-) |

5ASA: 5-aminosalicylate; ARB: Angiotensin II receptor blocker; HZ: Herpes zoster; OAD: Oral antiviral drug; pMS: Partial Mayo Score; PPI: Proton pump inhibitor; TNF-α: Tumor necrosis factor α; TOF: Tofacitinib.

group[7]. Although our results appear to be better than these results, the OCTAVE trials included endoscopic results and had a more stringent definition of efficacy, unlike real-world studies, which may explain this discrepancy.

Predictors of clinical response were investigated in other studies. Honap *et al*[22] reported that a younger age (OR: 1.04, 95%CI: 1.01-1.07) and higher CRP (OR: 0.292, 95%CI: 0.121-0.655) at baseline were associated with non-response at week 8. Biemans *et al*[25] reported that prior exposure to vedolizumab (OR: 0.327, 95%CI: 0.100-0.907) and SCCAI per point (OR: 0.825, 95%CI: 0.686-0.992) were associated with a reduced corticosteroid-free clinical remission rate at week 24. In the present study, we showed that a higher pMS at baseline was associated with non-responders at week 8 in multivariate analysis (OR: 0.61, 95%CI: 0.45-0.82, $P = 0.001$). Patients with higher activity tend to have a lower drug persistence rate, so it is more beneficial for patients with lower activity. Also, we revealed that patients who achieved clinical remission at week 8 continued TOF for a longer time than those who did not. The proportion of patients who had previously failed an anti-TNF-α agent in our study was lower than in the other real-world studies. Drug persistence was not significantly different between anti-TNF-α agent-naive and -failure patients, but the cumulative remission rate tended to be higher in patients who had previously failed anti-TNF-α agents.

In our study, although the relapse rate after tapering TOF was 46%, the proportion of patients who achieved a clinical remission after dose re-escalation was 57% at week 4 and 69% at week 12. Sands *et al*[26] reported that 35.1% and 49.1% of patients after dose escalation recaptured clinical remission at months 2 and 12, strongly suggesting that efficacy may be recaptured by dose re-escalation and making it a possible option for patients undergoing relapse.

With regard to safety, the most common adverse events in our study were hypercholesterolemia and infectious diseases such as HZ and upper respiratory tract inflammation. It was previously reported that the risk of HZ is increased in UC patients receiving TOF, especially in the elderly, Asians, and patients who previously failed anti-TNF-α agents[14]. The overall cohort (phase II and OCTAVE Induction 1 and 2, OCTAVE Sustain, OCTAVE Open) reported that 5.6% of patients developed HZ during TOF use[14]. In the present study, 6 (5.4%) patients developed HZ, a higher percentage than previously reported in Europe[23,25]. All patients were treated with oral antiviral therapy, but the oldest patient (68 years) developed postherpetic neuralgia. One patient switched to alternative therapy because of an insufficient clinical

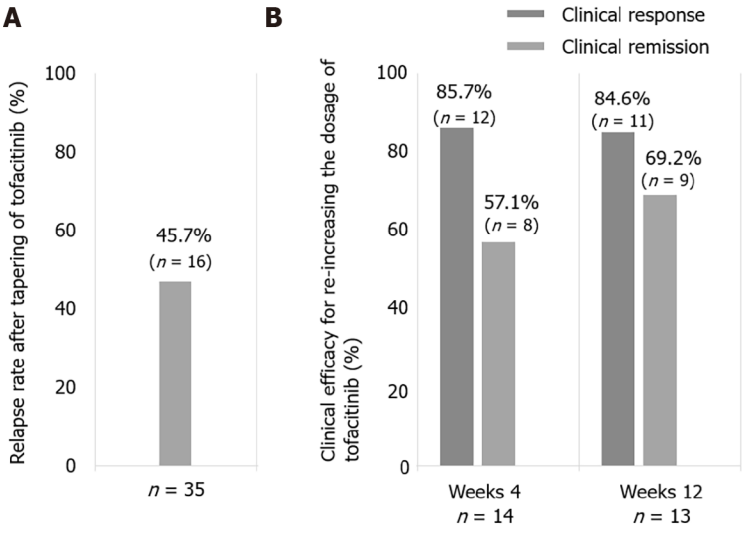


**Figure 7 Relapse rate and Clinical efficacy for re-increasing the dosage of tofacitinib.** A: Relapse rate after tapering of tofacitinib; B: Clinical efficacy for re-increasing the dosage of tofacitinib due to relapse after tapering of tofacitinib at weeks 4 and 12.

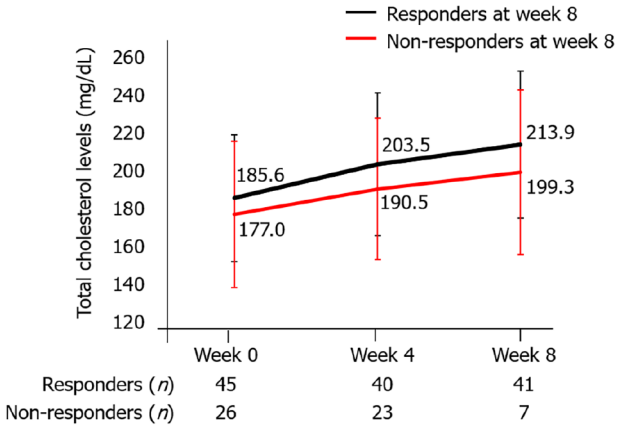


**Figure 8 Total cholesterol levels from baseline to week 8 between responders and non-responders at week 8.** Data represent the mean and standard deviation.

response, and others continued TOF after recovering from HZ.

Lipid levels reversibly increase during TOF administration in UC patients as well as in patients with rheumatoid arthritis and psoriatic arthritis[27-29]. Consistent with this finding, in our study, total cholesterol levels were significantly higher at week 4 and week 8 compared with baseline, but no severe cardiovascular events were observed. IBD patients have a higher risk of venous thromboembolism events compared with non-IBD patients, with a relative risk of 2.27[30]. In a United States Food and Drug Administration post-marketing requirement safety study including patients with rheumatoid arthritis aged at least 50 years old with at least 1 cardiovascular risk factor, patients treated with 10 mg TOF twice daily had a higher frequency of pulmonary embolism and death than patients treated with 5 mg TOF twice daily or with anti-TNF-α agent[31].

The present study has several limitations. First, the study was conducted retrospectively at a single center, with the inherent risk of bias and data missing for some patients. There is a possibility of selection bias to administrate TOF by each physician. Second, as endoscopy was not mandatory at baseline and follow-up, endoscopic data were only available in a small number of patients, and efficacy was judged only on the basis of clinical symptoms, so the evaluation may be insufficient. A strength of our study is that it is a large real-world study in an Asian population that demonstrated the efficacy and safety including related clinical needs of TOF. Shin *et al*[16] reported also analyzed more than 100 patients of Asian UC treated with TOF, but they did not analyze the efficacy of TOF according to the history of anti-TNF-α agent failure, relapse rate at dose reduction and the efficacy at dose re-escalation. In addition, while most of the included patients in the other studies had previously failed anti-TNF-α agents, only 62% of anti-TNF-α failure patients were included in our study. Therefore, we were able to determine whether previous treatment with anti-TNF-α agents affected the TOF efficacy. We analyzed for the first time in more than 100 Asian UC patients that the efficacy of TOF was effective irrespective to prior history of anti-TNF-α agents, relapse rate after tapering of TOF and effective rate for reinduction of TOF. These data is essential for clinical strategy of TOF treatment. The observation period of present study was one year, but we are planning to report further data for 3-year follow-up in the near future.

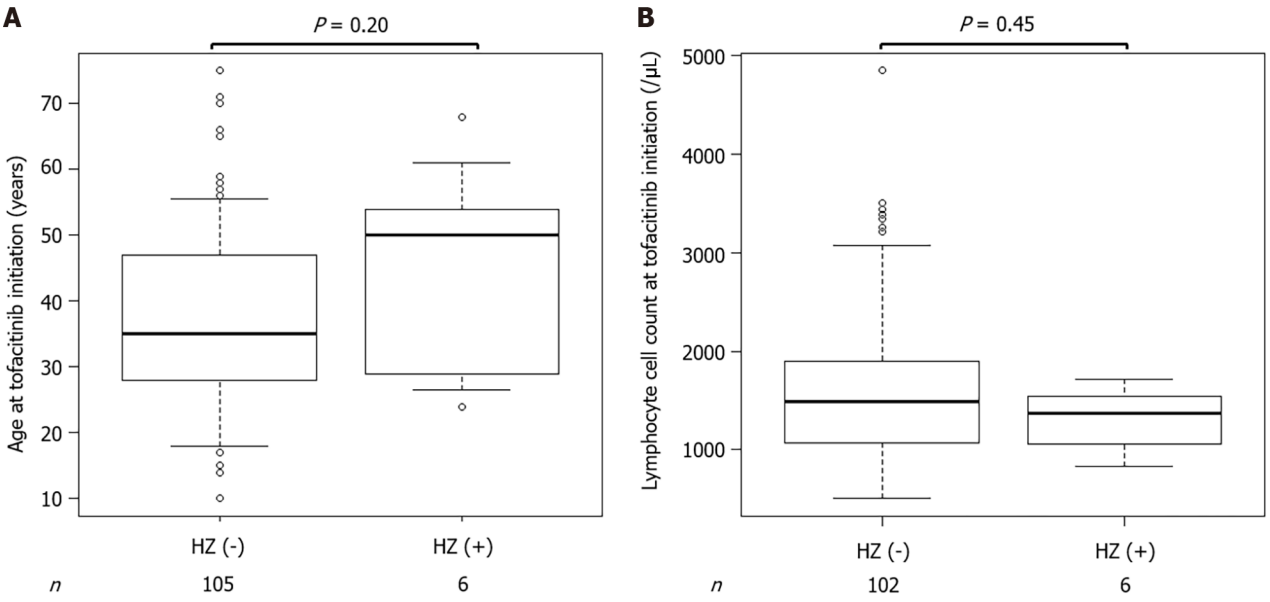


**Figure 9 Comparison of patient characteristics with and without herpes zoster.** A: Comparison of patient age at tofacitinib initiation between those who developed herpes zoster and those who did not; B: Comparison of the lymphocyte cell count at tofacitinib initiation between patients who developed herpes zoster and those who did not. HZ: Herpes zoster.

## CONCLUSION

In conclusion, TOF is more effective in low-activity UC patients in real practice and its efficacy is not affected by previous treatment with anti-TNF-α agents. Most patients in the clinical remission group at week 8 could continue TOF over a long follow-up period. Relapse occurred in 45.7% of patients after tapering TOF, but 85.7% of those patients recaptured a response to TOF by week 4. Although most patients continue TOF without severe adverse events, careful monitoring for HZ is necessary. Further studies are needed to investigate predictive factors for a response to TOF treatment and the positioning of TOF in many treatment options for active UC.

## ARTICLE HIGHLIGHTS

### *Research background*

Various therapeutic agents are currently available as advanced therapies. Tofacitinib (TOF) was approved to treat the patients with intractable ulcerative colitis (UC) as first Janus kinase inhibitor in Japan.

### *Research motivation*

Real-world data for the efficacy and safety of TOF treatment covering a period of more than 1 year with a sufficient number of Asian patients with UC are scarce. We investigated to optimize TOF treatment by using data of our specialized IBD center.

### *Research objectives*

The aim of this study is to investigate the efficacy and safety of TOF treatment covering a period of more than 1 year in patients with intractable UC.

### *Research methods*

We performed a retrospective single-center observational analysis of 111 UC patients administered TOF between May 2018 and February 2020. The primary outcome was the clinical response rate at week 8.

### *Research results*

The overall cumulative clinical remission rate was 61.7% at week 48 and history of anti- tumor necrosis factor-alpha (TNF-α) agents use had no influence ($P$ = 0.25). Baseline partial Mayo Score was significantly lower in responders compared with non-responders at week 8 (odds ratio: 0.61, 95% confidence interval: 0.45-0.82, $P$ = 0.001). Relapse occurred in 45.7% of patients after TOF tapering, and 85.7% of patients responded within 4 wk after re-increase.

### *Research conclusions*

TOF was more effective in UC patients with mild activity at baseline and its efficacy was not affected by previous

treatment with anti-TNF-α agents. Although careful monitoring for herpes zoster is necessary, most patients continue TOF without severe adverse events.

### *Research perspectives*

Future prospective studies with a large number of UC patients and long follow-up periods are needed in clinical practice and we should consider the positioning of TOF in many treatment options for active UC.

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## FOOTNOTES

**Author contributions:** Kojima K and Watanabe K participated in the conception and design of the study and were involved in the acquisition, analysis, or interpretation of data; Kojima K wrote the manuscript; Watanabe K and Shinzaki S accessed and verified the study data. All authors critically reviewed and provided final approval of the manuscript; all authors were responsible for the decision to submit the manuscript for publication.

**Institutional review board statement:** This investigation was approved by the Institutional Ethics Committee of Hyogo Medical University Hospital, No. 3030.

**Informed consent statement:** The need for patient consent was waived due to the retrospective nature of the study.

**Conflict-of-interest statement:** Watanabe K received honoraria and had expenses paid to attend or give a presentation or advice at a meeting for the following companies: AbbVie GK, EA Pharma Co., Ltd., Pfizer Japan Inc., Takeda Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Kyorin Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., Kissei Pharmaceutical Co., Ltd.; received research grants from EA Pharma Co., Ltd., Takeda Pharmaceutical Co., Ltd., EP-CRSU Co., Ltd., received scholarship grants from AbbVie GK, EA Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corporation, JIMRO Co., Ltd., Nippon Kayaku Co., Ltd.; and has been an endowed chair for AbbVie GK, EA Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Zeria Pharmaceutical Co., Ltd., JIMRO Co., Ltd., Otsuka Pharmaceutical Factory, Inc., Asahi Kasei Medical Co., Ltd., Mochida Pharmaceutical Co., Ltd. SS received honoraria and had expenses paid to attend or give a presentation or advice at meetings for AbbVie GK, EA Pharma Co., Ltd., Pfizer Japan Inc., Takeda Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Kyorin Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., Kissei Pharmaceutical Co., Ltd., Janssen Pharmaceutical K.K., Nippon Kayaku Co., Ltd., Gilead Sciences, JIMRO Co., Ltd., Nippon Kayaku Co., Zeria Pharmaceutical Co., Ltd., Alfresa Pharma Corporation, Astra Zeneka K.K., Eisai Co., Ltd., Sekisui Medical Co., Ltd. And received research grants from Sekisui Medical Co., Ltd.

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**Country/Territory of origin:** Japan

**ORCID number:** Kentaro Kojima 0000-0002-9000-2426; Kenji Watanabe 0000-0002-3781-4724; Mikio Kawai 0000-0001-8004-5927; Soichi Yagi 0000-0002-2454-2950; Koji Kaku 0000-0003-0988-8897; Maiko Ikenouchi 0000-0001-5816-5789; Toshiyuki Sato 0000-0001-8307-2445; Koji Kamikozuru 0000-0002-5474-3008; Yoko Yokoyama 0000-0002-2126-3489; Tetsuya Takagawa 0000-0001-7335-6962; Masahito Shimizu 0000-0002-1151-2058; Shinichiro Shinzaki 0000-0002-7051-618X.

**Corresponding Author's Membership in Professional Societies:** American College of Gastroenterology; American Gastroenterological Association; American Society for Gastrointestinal Endoscopy; The Japanese Society of Gastroenterology; European Crohn's and Colitis Organization; Asian Organization for Crohn's and Colitis; Japanese Society of Internal Medicine.

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ORIGINAL ARTICLE

Retrospective Study

# Novel subtype of obesity influencing the outcomes of sleeve gastrectomy: Familial aggregation of obesity

Ze-Yu Wang, Yun-Fei Qu, Tian-Ming Yu, Zeng-Lin Liu, Yu-Gang Cheng, Ming-Wei Zhong, San-Yuan Hu

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**Ze-Yu Wang, Yun-Fei Qu, Zeng-Lin Liu, Yu-Gang Cheng, Ming-Wei Zhong, San-Yuan Hu,** Department of General Surgery, The First Affiliated Hospital of Shandong First Medical University and Shandong Provincial Qianfoshan Hospital, Jinan 250000, Shandong Province, China

**Ze-Yu Wang, Yun-Fei Qu,** Department of Postgraduate, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan 250000, Shandong Province, China

**Tian-Ming Yu, Zeng-Lin Liu,** Department of General Surgery, Shandong Provincial Qianfoshan Hospital, Cheeloo College of Medicine Shandong University, Jinan 250000, Shandong Province, China

**Ming-Wei Zhong,** State Key Laboratory of Integration and Innovation of Classic Formula and Modern Chinese Medicine, Lunan Pharmaceutical Group Co. Ltd., Linyi 276005, Shandong Province, China

**Corresponding author:** Ming-Wei Zhong, MD, Professor, Department of General Surgery, The First Affiliated Hospital of Shandong First Medical University and Shandong Provincial Qianfoshan Hospital, No. 16766, Jingshi Road, Jinan 250000, Shandong Province, China.
zmwgz@126.com

## Abstract

### *BACKGROUND*

Differences in the preoperative characteristics and weight loss outcomes after sleeve gastrectomy (SG) between patients with familial aggregation of obesity (FAO) and patients with sporadic obesity (SO) have not been elucidated.

### *AIM*

To explore the impact of SG on weight loss and the alleviation of obesity-related comorbidities in individuals with FAO.

### *METHODS*

A total of 193 patients with obesity who underwent SG were selected. Patients with FAO/SO were matched 1:1 by propensity score matching and were categorized into 4 groups based on the number of first-degree relatives with obesity ($^1$SO *vs* $^1$FAO, $^2$SO *vs* $^2$FAO). The baseline characteristics, weight loss outcomes, prevalence of obesity-related comorbidities and incidence of major surgery-related complications were compared between groups.

***RESULTS***

We defined FAO as the presence of two or more first-degree relatives with obesity. Patients with FAO did not initially show significant differences in baseline data, short-term postoperative weight loss, or obesity-related comorbidities when compared to patients with SO preoperatively. However, distinctions between the two groups became evident at the two-year mark, with statistically significant differences in both percentage of total weight loss ($P$ = 0.006) and percentage of excess weight loss ($P$ < 0.001). The FAO group exhibited weaker remission of type 2 diabetes mellitus (T2DM) ($P$ = 0.031), hyperlipidemia ($P$ = 0.012), and non-alcoholic fatty liver disease (NAFLD) ($P$ = 0.003) as well as a lower incidence of acid reflux ($P$ = 0.038).

***CONCLUSION***

FAO patients is associated with decreased mid-to-long-term weight loss outcomes; the alleviation of T2DM, hyperlipidemia and NAFLD; and decreased incidence of acid reflux postoperatively.

**Key Words:** Obesity; Bariatric surgery; Sleeve gastrectomy; Family history; Weight loss



**Core Tip:** This was a retrospective study. We aimed to compare preoperative characteristics and postoperative outcomes between patients with familial aggregation of obesity (FAO) and those with sporadic obesity. The following data were examined: Baseline characteristics, weight changes at postoperative intervals (1, 3, 6, 12, 24, and 36 months), alleviation of obesity-related complications, and the occurrence of surgery-related complications. Such a comparative analysis provides valuable insights for guiding postoperative treatment and health education tailored to individuals with FAO.

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## INTRODUCTION

The prevalence of overweight and obesity is steadily increasing in China[1], making it the country with the largest population of overweight and obese individuals globally. It is expected that the prevalence of overweight [body mass index (BMI) 24.0-28.0 kg/m²] and obesity (BMI ≥ 28.0 kg/m²) in adults may reach 65.3% by 2030[2], thus leading to a significant public health concern[3]. Obesity can lead to a myriad of multisystem abnormalities, encompassing cardiovascular disease, type 2 diabetes mellitus (T2DM), hyperlipidemia, hyperuricemia, nonalcoholic fatty liver disease, polycystic ovary syndrome, mental disorders, locomotor and joint disorders, and respiratory diseases, among other comorbidities.

Obesity results from the intricate interplay of genetic, environmental, lifestyle, and sociocultural factors[4]. These factors affect fat accumulation or consumption by influencing several physiologic mediators of food intake and energy expenditure[5]. An individual's family–often representing a microcosm of closely aligned genetic profiles, lifestyle behaviors, environmental exposures, and sociocultural outlooks–exerts a substantial influence on the emergence of obesity and the outcomes of weight management. Sleeve gastrectomy (SG), which accounted for 84.9% of bariatric surgical procedures[6], effectively facilitates weight loss in patients with obesity while markedly enhancing metabolic processes and ameliorating obesity-related comorbidities[7].

The familial aggregation of diseases is a focal point of research across several disciplines, including psychiatry[8], neurology[9] and oncology[10,11]. By studying the characteristics of first-degree relatives of individuals with obesity, we can gain deeper insights into the pathogenesis of obesity, thereby contributing to the search for novel treatments or prevention methods[12]. This study provides a theoretical foundation for precision prevention and treatment of obesity.

## MATERIALS AND METHODS

### *Patients*

We selected a cohort of 193 patients with obesity who met the criteria for SG and who underwent surgery at our medical center between December 2019 and April 2023 for this observational study. Following surgery, all patients received uniform postoperative guidance and health education.

### Inclusion/exclusion criteria

**Inclusion criteria:** (1) Patients aged between 16 and 65 years; (2) patients met the surgical indications outlined in the Chinese Guidelines for the Surgical Management of Obesity and T2DM (2019 edition)[13]; and (3) patients were capable of undergoing normal follow-up after SG.

**Exclusion criteria:** (1) Patients lacking information about first-degree relatives; (2) patients requiring obesity-inducing medications for their medical conditions after the operation; (3) patients who became pregnant shortly after the procedure; and (4) patients on appetite suppressants (such as metformin) following surgery.

### Grouping method

The familial aggregation of obesity (FAO) group and the sporadic obesity (SO) group: We divided the participants into groups according to the number of first-degree relatives (parents, children and siblings of the proband) with obesity (BMI > 28 kg/m$^2$). The grouping criteria for FAO were as follows.

$^1$FAO (FAO group 1): Except for the proband, the number of first-degree relatives with obesity ≥ 1. $^1$SO (SO group 1): Except for the proband, the number of first-degree relatives with obesity = 0.

$^2$FAO (FAO group 2): Except for the proband, the number of first-degree relatives with obesity ≥ 2. $^2$SO (SO group 2): Except for the proband, the number of first-degree relatives with obesity < 2.

### Data collection

The data were collected independently by two individuals. Perioperative data for all patients, including sex, age, BMI, family history, waist circumference, hip circumference, and obesity-related comorbidities (such as hypertension, T2DM, and hyperlipidemia), were systematically recorded using the electronic case management system. Following SG, we conducted thorough postoperative follow-ups at 1, 3, 6, 12, 24, and 36 months through a combination of hospital visits and telephone interviews. These follow-ups involved evaluating postoperative weight, conducting blood tests, assessing surgery-related complications, and monitoring the improvement of preoperative obesity-related comorbidities. To assess the effectiveness of weight loss surgery, we employed the percentage of total weight loss (%TWL) and percentage of excess weight loss (%EWL) as evaluation criteria.

%TWL = (initial body weight - final body weight)/initial weight × 100%.

%EWL = [(initial body weight - final body weight)/(initial weight - ideal body weight)] × 100%.

Ideal BMI (IBMI): 23 kg/m$^2$ (Asian standard), ideal body weight: IBMI × (height)$^2$.

### Statistical methods

All clinical data were analyzed using SPSS statistical software (version 26.0; SPSS, Inc., Chicago, IL, United States). Propensity score matching (PSM)[14] was employed for 1:1 matching of FAO/SO groups. Independent samples *t* tests were also conducted to compare preoperative baseline data and postoperative weight loss outcomes at each follow-up interval between patients in the FAO group and the SO group. Linear regression was employed to identify factors influencing %TWL and %EWL. To compare the prevalence of obesity-related comorbidities and surgery-related complications in patients in the FAO and SO groups, $\chi^2$ or Fisher's exact test was employed both preoperatively and at the 6-month postoperative assessment. *P* values < 0.05 indicated statistical significance.

## RESULTS

### Baseline patient characteristics

This observational study included a total of 193 patients who underwent SG (male: 64, 33.2%; female: 129, 66.8%), with a mean BMI of 41.3 ± 7.0 kg/m$^2$. Among the obese patients, various obesity-related comorbidities were prevalent, including metabolic syndrome (88, 45.6%), hypertension (67, 34.7%), T2DM (94, 48.7%), hyperlipidemia (81, 42%), sleep apnea hypopnea syndrome (128, 66.3%), polycystic ovary syndrome (31, 24.0%, *n* = 129), nonalcoholic fatty liver disease (163, 84.5%), gout (10, 5.2%), and hyperuricemia (114, 59.1%). Additionally, 113 patients (58.5%) were in the $^1$FAO group, while 58 (30.0%) were in the $^2$FAO group. Specific indicators of obesity-related comorbidities are detailed in Table 1.

### Comparison of preoperative information

**Preoperative baseline information:** We applied PSM analysis to pair patients in the $^1$SO/$^1$FAO and $^2$SO/$^2$FAO groups utilizing predictors of major obesity-related comorbidities (metabolic syndrome, hypertension, T2DM, hyperlipidemia, and nonalcoholic fatty liver disease). The matched groups exhibited no significant differences in patient age, height, weight, or BMI, as shown in Table 2.

**Preoperative obesity-related comorbidities:** We conducted PSM analysis again to compare patients within the matched $^1$SO/$^1$FAO and $^2$SO/$^2$FAO groups utilizing sex and BMI as predictors. The analysis revealed no significant differences in preoperative obesity-related comorbidities between patients in the matched groups, as indicated in Table 3.

**Comparison of postoperative information:** All 193 patients completed 1/3/6 months of postoperative follow-up, 107 patients completed 12 months of postoperative follow-up, 60 patients completed 24 months of postoperative follow-up, and 21 patients completed 36 months of postoperative follow-up (analysis at 36 months was primarily focused on trend interpretation).

**Table 1 Preoperative data of all patients (*n* = 193): Baseline information and obesity-related comorbidities, *n* (%)**

| Baseline | Total, *n* = 193 |
|---|---|
| Sex (Females) | 129 (66.8) |
| Age (yr, mean ± SD) | 31.5 ± 8.2 |
| Height (cm, mean ± SD) | 169.3 ± 8.0 |
| Weight (kg, mean ± SD) | 119.6 ± 27.7 |
| Body mass index (kg/m$^2$, mean ± SD) | 41.3 ± 7.0 |
| Waistline (cm, mean ± SD) | 123.2 ± 17.2 |
| Hipline (cm, mean ± SD) | 129.4 ± 15.7 |
| $^1$FAO | 113 (58.5) |
| $^2$FAO | 58 (30.0) |
| **Obesity-related comorbidities** | |
| Metabolic syndrome | 88 (45.6) |
| Hypertension | 67 (34.7) |
| Cardiovascular disease | 10 (5.2) |
| Type 2 diabetes mellitus | 94 (48.7) |
| Impaired glucose tolerance | 78 (40.4) |
| Hyperlipoidemia | 81 (42.0) |
| Obstructive sleep apnea | 128 (66.3) |
| Polycystic ovarian syndrome (*n* = 129) | 31 (24.0) |
| Non-alcoholic fatty liver disease | 163 (84.5) |
| Gout | 10 (5.2) |
| Hyperuricemia | 114 (59.1) |

The data in the table are *n* (%) or mean ± SD. FAO: Familial aggregation of obesity.

**Table 2 Preoperative baseline information**

| | $^1$SO, *n* = 75 | $^1$FAO, *n* = 75 | *P* value | $^2$SO, *n* = 54 | $^2$FAO, *n* = 54 | *P* value |
|---|---|---|---|---|---|---|
| Sex [female, *n* (%)] | 50 (66.7) | 48 (64.0) | 0.731 | 36 (66.7) | 32 (59.3) | 0.425 |
| Age (yr) | 29.5 ± 6.8 | 31.8 ± 9.1 | 0.234 | 31.7 ± 8.6 | 30.7 ± 8.6 | 0.548 |
| Height (cm) | 170.3 ± 7.9 | 170.1 ± 7.7 | 0.884 | 169.9 ± 7.2 | 170.4 ± 8.3 | 0.711 |
| Body Weight (kg) | 123.5 ± 30.4 | 122.2 ± 29.9 | 0.840 | 120.9 ± 28.6 | 124.2 ± 31.3 | 0.562 |
| BMI (kg/m$^2$) | 42.2 ± 8.1 | 41.8 ± 7.5 | 0.795 | 41.5 ± 7.4 | 42.3 ± 7.9 | 0.591 |
| Waistline (cm) | 126.0 ± 18.3 | 124.1 ± 19.9 | 0.731 | 124.8 ± 19.8 | 124.3 ± 19.4 | 0.916 |
| Hipline (cm) | 128.3 ± 12.3 | 129.8 ± 16.5 | 0.744 | 128.0 ± 13.3 | 130.7 ± 17.4 | 0.459 |

The data in the table are *n* (%) or mean ± SD. SO: Sporadic obesity; FAO: Familial aggregation of obesity; BMI: Body mass index.

We conducted PSM analysis to align patients in the $^1$SO/$^1$FAO and $^2$SO/$^2$FAO groups. We employed sex, preoperative BMI, and major obesity-related comorbidities (metabolic syndrome, hypertension, T2DM, hyperlipidemia, and nonalcoholic fatty liver disease) as predictors to minimize differences between the groups and mitigate the impact of variations in these factors on surgical outcomes.

After PSM analysis, the patient counts were as follows:

$^1$SO *vs* $^1$FAO = 73 *vs* 73; $^2$SO *vs* $^2$FAO = 53 *vs* 53 (1/3/6 months after surgery).

$^1$SO *vs* $^1$FAO = 37 *vs* 43; $^2$SO *vs* $^2$FAO = 52 *vs* 31 (12 months after surgery).

**Table 3 Preoperative obesity-related comorbidities, *n* (%)**

| | | *n* | [1]SO, *n* = 80 | [1]FAO, *n* = 80 | $\chi^2$ | *P* value | *n* | [2]SO, *n* = 58 | [2]FAO, *n* = 58 | $\chi^2$ | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MS | Without | 89 | 43 (53.8) | 46 (57.5) | 0.228 | 0.633 | 67 | 34 (58.6) | 33 (56.9) | 0.035 | 0.851 |
| | With | 71 | 37 (46.3) | 34 (42.5) | | | 49 | 24 (41.4) | 25 (43.1) | | |
| HTN | Without | 105 | 54 (67.5) | 51 (63.8) | 0.249 | 0.618 | 75 | 33 (56.9) | 42 (72.4) | 3.056 | 0.080 |
| | With | 55 | 26 (32.5) | 29 (36.3) | | | 41 | 25 (43.1) | 16 (27.6) | | |
| T2DM | Without | 86 | 43 (53.8) | 43 (53.8) | | > 0.999 | 59 | 31 (53.4) | 28 (48.3) | 0.310 | 0.577 |
| | With | 74 | 37 (46.3) | 37 (46.3) | | | 57 | 27 (46.6) | 30 (51.7) | | |
| IGT | Without | 89 | 45 (56.3) | 44 (55.0) | 0.025 | 0.874 | 71 | 33 (56.9) | 38 (65.5) | 0.908 | 0.341 |
| | With | 71 | 35 (43.8) | 36 (45.0) | | | 45 | 25 (43.1) | 20 (34.5) | | |
| HLP | Without | 96 | 51 (63.8) | 45 (56.3) | 0.938 | 0.333 | 68 | 39 (67.2) | 29 (50.0) | 3.554 | 0.059 |
| | With | 64 | 29 (36.3) | 35 (43.8) | | | 48 | 19 (32.8) | 29 (50.0) | | |
| PCOS | Without | 87 | 41 (74.5) | 46 (80.7) | 0.612 | 0.434 | 52 | 27 (77.1) | 25 (73.5) | 0.157 | 0.924 |
| | With | 25 | 14 (25.5) | 11 (19.3) | | | 17 | 8 (22.9) | 9 (26.5) | | |
| NAFLD | Without | 26 | 16 (20.0) | 10 (12.5) | 1.653 | 0.199 | 19 | 8 (13.8) | 11 (19.0) | 0.566 | 0.452 |
| | With | 134 | 64 (80.0) | 70 (87.5) | | | 97 | 50 (86.2) | 47 (81.0) | | |
| OSA | Without | 53 | 27 (33.8) | 26 (32.5) | 0.028 | 0.867 | 37 | 14 (24.1) | 23 (39.7) | 3.215 | 0.073 |
| | With | 107 | 53 (66.3) | 54 (67.5) | | | 79 | 44 (75.9) | 35 (60.3) | | |
| HUA | Without | 68 | 37 (46.3) | 31 (38.8) | 0.921 | 0.337 | 42 | 18 (31.0) | 24 (41.4) | 1.344 | 0.246 |
| | With | 92 | 43 (53.8) | 49 (61.3) | | | 74 | 40 (69.0) | 34 (58.6) | | |

Only female participants were analyzed to compare polycystic ovarian syndrome prevalence. The number of females is 112 [[1]sporadic obesity (SO) *vs* [1] familial aggregation of obesity (FAO) = 55 *vs* 57] and 69 ([2]SO *vs* [2]FAO = 35 *vs* 34). SO: Sporadic obesity; FAO: Familial aggregation of obesity; MS: Metabolic syndrome; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; IGT: Impaired glucose tolerance; HLP: Hyperlipoidemia; PCOS: Polycystic ovarian syndrome; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive sleep apnea; HUA: Hyperuricemia.

[1]SO *vs* [1]FAO = 22 *vs* 22; [2]SO *vs* [2]FAO = 37 *vs* 17 (24 months after surgery).
[1]SO *vs* [1]FAO = 6 *vs* 7; [2]SO *vs* [2]FAO = 11 *vs* 8 (36 months after surgery).

**Weight loss:** (1) SG results in a substantial weight reduction in the majority of patients after the procedure, as shown in Table 4. There was no significant difference in short-term postoperative weight loss between patients in the [1]FAO group and those in the [1]SO group. Nevertheless, over time, notable differences became evident at 24 months postsurgery, with patients in the [1]FAO group experiencing less weight loss after SG than their counterparts in the [1]SO group (%TWL: $P$ = 0.025; %EWL: $P$ = 0.025). Comparatively, patients in the [2]FAO group exhibited similar but more pronounced differences than did those in the [2]SO group (BMI: $P$ = 0.003, %TWL: $P$ = 0.006, %EWL: $P$ < 0.001). Several line graphs are shown in Figure 1. These lines of view visually illustrate the difference above. Patients with FAO regain weight to some extent at the two-year postoperative mark, while patients with SO are able to maintain a more favorable weight loss outcome.

And (2) Multiple linear regression analysis. To further explore the factors affecting weight loss outcomes and assess the impact of FAO, we conducted linear regression analyses on %TWL and %EWL at various postoperative time points (Tables 5 and 6). The %TWL, %EWL and BMI exhibited normal distributions. Factors affecting %TWL and %EWL showed no significant multicollinearity. After controlling for the effects of age and obesity-related comorbidities on surgery, we observed that the impact of [1]FAO on weight loss outcomes was not significantly different at 24 months postsurgery, whereas [2]FAO and preoperative BMI exhibited statistically significant differences in their influence on weight loss outcomes, as indicated in Tables 5 and 6 (%TWL: [2]FAO: $P$ < 0.001, BMI: $P$ = 0.001; %EWL: [2]FAO: $P$ < 0.001).

**Alleviation of obesity-related comorbidities**: SG significantly alleviates a wide range of obesity-related comorbidities, including metabolic syndrome, hypertension, T2DM, hyperlipoidemia (HLP), non-alcoholic fatty liver disease (NAFLD), and hyperuricemia, in the majority of patients 6 months postsurgery. Nevertheless, the extent of remission varies between patients with SO or FAO. As shown in Table 7, the incidence of NAFLD was greater in the [1]FAO group than in the [1]SO group ($P$ = 0.015). The [2]FAO group exhibited a higher prevalence of T2DM ($P$ = 0.031), HLP ($P$ = 0.012), and NAFLD ($P$ = 0.003) than the [2]SO group.

**Surgery-related complications**: We compared major surgery-related comorbidities (acid reflux, nausea/vomiting, alopecia, and constipation) postsurgery among the different groups of patients. There was no significant difference in

**Table 4 Postoperative body mass index, total weight loss percentage, excess weight loss percentage**

| | | [1]SO, *n* = 73 | [1]FAO, *n* = 73 | *P* value | [2]SO, *n* = 53 | [2]FAO, *n* = 53 | *P* value |
|---|---|---|---|---|---|---|---|
| Baseline | Sex [female, *n* (%)] | 49 (67.1) | 49 (67.1) | > 0.999 | 36 (67.9) | 33 (62.3) | 0.541 |
| | Age (yr) | 30.5 ± 7.7 | 31.0 ± 8.5 | 0.706 | 31.6 ± 8.7 | 30.6 ± 8.3 | 0.537 |
| | Height (cm) | 169.2 ± 8.4 | 169.9 ± 8.1 | 0.617 | 169.8 ± 7.5 | 170.2 ± 8.5 | 0.817 |
| | Body weight (kg) | 118.8 ± 26.7 | 120.7 ± 27.4 | 0.660 | 123.2 ± 27.3 | 123.2 ± 31.3 | 0.995 |
| | BMI (kg/m$^2$) | 41.2 ± 6.9 | 41.5 ± 6.8 | 0.802 | 42.4 ± 6.9 | 42.1 ± 7.9 | 0.842 |
| | Waistline (cm) | 123.0 ± 16.0 | 123.4 ± 17.3 | 0.892 | 124.9 ± 18.4 | 123.4 ± 19.3 | 0.725 |
| | Hipline (cm) | 128.8 ± 16.3 | 131.0 ± 14.4 | 0.439 | 131.0 ± 18.1 | 129.8 ± 17.1 | 0.771 |
| BMI | Pre-op (kg/m$^2$) | 41.2 ± 6.9 | 41.5 ± 6.8 | 0.802 | 42.4 ± 6.9 | 42.1 ± 7.9 | 0.842 |
| | 1 month (kg/m$^2$) | 35.5 ± 5.9 | 36.2 ± 6.3 | 0.465 | 36.9 ± 5.7 | 36.9 ± 7.6 | 0.974 |
| | 3 months (kg/m$^2$) | 31.5 ± 5.4 | 32.4 ± 5.6 | 0.364 | 32.9 ± 5.3 | 33.0 ± 6.6 | 0.950 |
| | 6 months (kg/m$^2$) | 28.4 ± 4.9 | 29.3 ± 5.0 | 0.246 | 29.6 ± 4.9 | 29.9 ± 5.9 | 0.784 |
| | 12 months (kg/m$^2$) | 26.9 ± 4.8 | 27.1 ± 3.9 | 0.830 | 27.2 ± 4.4 | 28.6 ± 5.5 | 0.213 |
| | 24 months (kg/m$^2$) | 26.3 ± 5.1 | 28.2 ± 4.2 | 0.201 | 26.9 ± 4.2 | 31.3 ± 5.7 | 0.003 |
| | 36 months (kg/m$^2$) | 26.9 ± 5.1 | 29.3 ± 5.3 | 0.428 | 27.5 ± 4.0 | 32.2 ± 6.2 | 0.061 |
| %TWL | 1 month | 13.8 ± 4.4 | 12.8 ± 3.2 | 0.116 | 12.7 ± 3.5 | 12.6 ± 3.9 | 0.896 |
| | 3 months | 23.2 ± 5.3 | 21.8 ± 5.3 | 0.112 | 22.2 ± 4.2 | 21.6 ± 6.2 | 0.571 |
| | 6 months | 30.9 ± 5.8 | 29.0 ± 6.8 | 0.072 | 29.9 ± 5.2 | 28.7 ± 7.2 | 0.323 |
| | 12 months | 36.1 ± 7.5 | 32.7 ± 8.3 | 0.063 | 35.6 ± 6.8 | 32.0 ± 9.8 | 0.081 |
| | 24 months | 37.4 ± 7.8 | 30.0 ± 12.6 | 0.025 | 36.6 ± 7.4 | 26.7 ± 12.5 | 0.006 |
| | 36 months | 41.3 ± 11.7 | 27.4 ± 10.5 | 0.044 | 37.0 ± 11.6 | 26.4 ± 13.3 | 0.079 |
| %EWL | 1 month | 33.2 ± 12.1 | 32.2 ± 16.9 | 0.675 | 29.1 ± 9.2 | 32.5 ± 19.9 | 0.262 |
| | 3 months | 56.5 ± 16.5 | 54.4 ± 25.5 | 0.561 | 51.8 ± 12.8 | 54.5 ± 30.0 | 0.541 |
| | 6 months | 74.9 ± 18.9 | 71.5 ± 28.9 | 0.399 | 69.8 ± 17.4 | 71.2 ± 32.5 | 0.771 |
| | 12 months | 84.9 ± 24.8 | 79.8 ± 20.8 | 0.322 | 82.7 ± 22.4 | 75.2 ± 25.0 | 0.162 |
| | 24 months | 89.1 ± 24.8 | 70.9 ± 27.0 | 0.025 | 83.9 ± 20.8 | 59.2 ± 25.2 | < 0.001 |
| | 36 months | 83.9 ± 25.1 | 66.7 ± 20.6 | 0.202 | 77.8 ± 22.8 | 56.9 ± 25.3 | 0.084 |

The data in the table are *n* (%) or mean ± SD. Analysis at 36 months is primarily focused on trend interpretation. SO: Sporadic obesity; FAO: Familial aggregation of obesity; BMI: Body mass index; %TWL: Total weight loss percentage; %EWL: Excess weight loss percentage; Pre-op: Pre-operation.

surgery-related complications between patients in the [1]FAO group and the [1]SO group ($P > 0.05$). However, the prevalence of acid reflux symptoms was lower in the [2]FAO group than in the [2]SO group ([2]SO:[2]FAO = 24.5%:9.4%, $P = 0.038$). There was no significant difference in nausea/vomiting, alopecia, or constipation between the two groups.

## DISCUSSION

Obesity and its severity are influenced primarily by genetic, environmental, lifestyle, and sociocultural factors[5]. Families, as fundamental units in the context of obesity, often share common genetic traits, lifestyle behaviors, and sociocultural perceptions. While many studies have focused on the family history of obesity in adolescents and children [4], there is a lack of research investigating the impact of FAO on SG.

Our study examined the impact of family history on patients with obesity and introduced the novel concept of FAO. After using PSM analysis to eliminate the possible influence of sex, preoperative BMI, and major obesity-related comorbidities on surgical outcomes, we found a significant difference in the weight loss outcomes of SG between patients with FAO, defined as two or more first-degree relatives with obesity, and those with SO. Specifically, patients with FAO experienced worse weight loss outcomes as well as lower remission rates of T2DM and NAFLD after SG. These findings suggest a potential association between FAO and weight regain after SG.

**Table 5 Factors affecting percentage total weight loss**

| | | 1 month | 3 months | 6 months | 12 months | 24 months |
|---|---|---|---|---|---|---|
| %TWL | [1]FAO | -1.036 | -1.352 | -1.838 | -1.542 | -5.123 |
| | Sex | -0.361 | -1.054 | -1.798 | -1.229 | -1.227 |
| | BMI | 0.0280 | 0.096 | 0.250[b] | 0.444[a] | 0.672[a] |
| | MS | 0.091 | -0.962 | -1.006 | -3.808 | 0.829 |
| | HTN | 0.876 | 0.864 | 0.082 | -0.844 | -5.728 |
| | T2DM | -0.370 | -0.283 | -0.087 | -0.376 | -3.427 |
| | HLP | 0.980 | 1.030 | -0.817 | -1.203 | -4.127 |
| | NAFLD | -0.517 | -0.410 | -1.568 | 0.065 | -0.958 |
| | OSA | -0.371 | 0.939 | 1.033 | 2.752 | 3.023 |
| | HUA | 0.506 | 0.028 | -0.039 | 2.115 | 2.590 |
| %TWL | [2]FAO | -0.272 | -0.671 | -1.385 | -3.164 | -9.486[b] |
| | Sex | 1.046 | 0.461 | 1.013 | 2.693 | 2.206 |
| | BMI | -0.039 | 0.054 | 0.156 | 0.285 | 0.618[a] |
| | MS | -0.891 | -0.860 | -1.852 | -1.634 | -4.611 |
| | HTN | 0.306 | 0.810 | -0.088 | -2.749 | -3.853 |
| | T2DM | -0.338 | -1.478 | -1.548 | 0.198 | -1.050 |
| | HLP | 1.318 | 1.867 | 0.690 | -2.605 | -0.980 |
| | NAFLD | -0.113 | -0.303 | 0.482 | 0.338 | 2.968 |
| | OSA | -0.483 | 0.747 | 0.288 | 2.558 | 0.332 |
| | HUA | -0.367 | -1.384 | -1.298 | 0.120 | -0.298 |

[a]$P < 0.01$.

[b]$P < 0.001$.

[1]SO *vs* [1]FAO: $VIF^{1}_{FAO}$ = 1.182, $VIF_{Sex}$ = 2.056, $VIF_{BMI}$ = 1.783, $VIF_{MS}$ = 3.514, $VIF_{HT}N$ = 2.561, $VIF_{T2DM}$ = 1.854, $VIF_{HLP}$ = 1.726, $VIF_{NAFLD}$ = 1.244, $VIF_{OSA}$ = 2.057, $VIF_{HUA}$ = 2.03. [2]SO *vs* [2]FAO: $VIF^{2}_{FAO}$ = 1.067, $VIF_{Sex}$ = 1.683, $VIF_{BMI}$ = 1.74, $VIF_{MS}$ = 3.553, $VIF_{HT}N$ = 1.663, $VIF_{T2DM}$ = 1.908, $VIF_{HLP}$ = 2.352, $VIF_{NAFLD}$ = 1.19, $VIF_{OSA}$ = 1.343, $VIF_{HUA}$ = 1.369. The data in the table are unstandardized coefficients (β-values). FAO: Familial aggregation of obesity; BMI: Body mass index; MS: Metabolic syndrome; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; HLP: Hyperlipoidemia; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive sleep apnea; HUA: Hyperuricemia; VIF: Variance inflation factor.

In terms of genetics, families of patients with FAO may share common obesity susceptibility genes. These genes included single-gene obesity genes, such as those encoding leptin (Lep) and its receptor (Lepr), the melanocortin-4 receptor, and proopiomelanocortin, and polygenic obesity genes (*FTO loci*), among others[5]. These genes influence weight by regulating the energy balance in the central nervous system, ultimately affecting body weight[15,16]. However, it is crucial to note that genetics alone cannot fully explain the differences in surgical outcomes between the two groups [17]. The disparities in surgical outcomes result from the combined influence of genetic and environmental factors.

In terms of environmental exposures, diet and lifestyle, patients who undergo SG and their family members share common obesity-inducing factors, such as similar dietary and exercise habits. All of these conditions exhibit many similarities, as both patients and their family members suffer from obesity and related comorbidities, which are often accompanied by a sedentary lifestyle[12]. In terms of cognition, similar cognitive levels within the family[18] determine the development of obesity and weight loss outcome of bariatric surgery. The combination of these factors results in weaker dietary and exercise maintenance abilities among patients with FAO[19] than in those with SO, possibly contributing to their mid-to-long-term postoperative weight regain.

SG significantly improves various metabolic processes[20], including glucose metabolism, lipid metabolism, and amino acid metabolism, in patients with obesity. Patients with FAO exhibit lower remission rates for T2DM, hyperlipidemia and NAFLD. This difference may be related to the extent of improvement in glucose and lipid metabolism. By aggregating information about patients with FAO, we aimed to investigate and identify factors influencing the postoperative remission of glucose and lipid metabolism. This research may lead to the use of novel therapeutic approaches for individuals with primary or secondary metabolic disorders.

The incidence of *de novo* gastroesophageal reflux disease (GORD) after SG is approximately 24.8%[21]. We observed a significantly lower incidence of postoperative acid reflux in patients with FAO than in those with SO. This difference may be associated with reduced intra-abdominal pressure[22]. The International Federation for the Surgery of Obesity and Metabolic Disorders recommends performing an endoscopy at 1 year after surgery, followed by subsequent screenings

**Table 6 Factors affecting percentage of excess weight loss**

| | | 1 month | 3 months | 6 months | 12 months | 24 months |
|---|---|---|---|---|---|---|
| %EWL | [1]FAO | -0.698 | -1.238 | -2.329 | -4.439 | -14.637 |
| | Sex | 3.531 | 4.486 | 2.864 | -4.092 | -1.340 |
| | BMI | -1.100 [c] | -1.751 [c] | -1.903 [c] | -1.397 [b] | -0.757 |
| | MS | -1.272 | -4.902 | -6.480 | -10.983 | 2.548 |
| | HTN | 4.388 | 5.435 | 4.388 | 1.123 | -13.696 |
| | T2DM | 2.464 | 3.318 | 4.445 | 0.240 | -11.171 |
| | HLP | 0.807 | 1.271 | -2.716 | -4.837 | -11.703 |
| | NAFLD | -5.720 | -8.292 | -11.392 [a] | 0.987 | 1.587 |
| | OSA | -2.937 | -0.133 | -0.533 | 5.991 | 6.384 |
| | HUA | 0.940 | -0.329 | -1.269 | 2.565 | 2.079 |
| %EWL | [2]FAO | 2.067 | 1.089 | -0.691 | -7.224 | -23.513 [c] |
| | Sex | 6.912 [a] | 8.730 | 10.880 | 5.487 | 7.279 |
| | BMI | -1.145 [c] | -1.806 [c] | -2.054 [c] | -1.539 [c] | -0.666 |
| | MS | -7.068 | -8.342 | -12.334 | -6.298 | -5.596 |
| | HTN | 5.569 | 9.484 | 8.283 | -2.062 | -8.179 |
| | T2DM | 3.291 | 0.925 | 0.717 | -0.222 | -10.684 |
| | HLP | 2.784 | 4.219 | 1.786 | -7.448 | -5.069 |
| | NAFLD | -3.588 | -6.218 | -5.107 | 1.598 | 7.874 |
| | OSA | -5.186 | -3.097 | -5.148 | 2.368 | -2.031 |
| | HUA | -2.054 | -5.344 | -5.365 | -2.837 | -5.233 |

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
The data in the table are unstandardized coefficients (β-values). FAO: Familial aggregation of obesity; BMI: Body mass index; MS: Metabolic syndrome; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; HLP: Hyperlipoidemia; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive sleep apnea; HUA: Hyperuricemia; %EWL: Percentage of excess weight loss; %TWL: Percentage of total weight loss.

every 2 to 3 years based on the results of the initial examination[23]. Our findings may further contribute to the precise prevention and treatment of postoperative *de novo* GORD.

Impaired family functioning may be one of the factors influencing surgical outcomes[24]. A bidirectional relationship exists between family members and patients. Family members can play a supportive role in assisting patients in achieving and sustaining weight loss[12]. The 'halo effect'[25] of patients extends to their family members, resulting in positive changes. This includes improvements in family members' dietary and lifestyle habits[26,27] and an enhancement in their quality of life[28]. Interventions targeting obesity, by incorporating a family systems framework, can also extend the benefits of surgery to the family members of individuals with obesity[29].

The concept of familial aggregation of diseases helps in identifying groups of individuals with shared disease characteristics. For instance, individuals with a family history of type 2 diabetes are more likely to experience overweight/obesity and are susceptible to adverse metabolic consequences of fat accumulation[30]. Patients with a family history of Alzheimer's disease may experience limitations in cognitive function improvement after SG[31]. Moreover, these findings could aid in identifying susceptibility genes for related diseases and gaining deeper insights into potential pathophysiological mechanisms[32], ultimately leading to the discovery of new preventive or therapeutic strategies for obesity[5]. Currently, large-scale genome-wide association studies have identified more than 1100 obesity-associated genetic loci[33]. This study offers a novel perspective. By studying families as units of investigation rather than isolated individuals, it is possible to further discover susceptibility genes for obesity, predict the development of obesity, and enhance strategies for diagnosing and treating obesity[34].

Limitations: (1) Based on our observational study, differences in patients with FAO gradually emerge only in the mid-to-long-term postsurgery. We are actively investigating longer-term surgical outcomes as part of our ongoing research; (2) We excluded a few patients for whom it was difficult to trace first-degree relative information (*e.g.*, adopted, stepparents, or deceased first-degree relatives). These patients exhibited weight loss results equal to or below the average, possibly due to impaired family functioning[24], posing challenges for detailed analysis. We intend to increase the sample size to further explore potential underlying factors; and (3) This study was conducted at a single center, acknowledging

**Table 7 Obesity-related comorbidities at 6 months postoperatively, *n* (%)**

| | | *n* | [1]SO, *n* = 73 | [1]FAO, *n* = 73 | $\chi^2$ | *P* value | *n* | [2]SO, *n* = 53 | [2]FAO, *n* = 53 | $\chi^2$ | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MS | Without | 133 | 65 (89) | 68 (93.2) | 0.760 | 0.383 | 100 | 52 (98.1) | 48 (90.6) | | 0.205 |
| | With | 13 | 8 (11.0) | 5 (6.8) | | | 6 | 1 (1.9) | 5 (9.4) | | |
| HTN | Without | 132 | 66 (90.4) | 66 (90.4) | | > 0.999 | 99 | 50 (94.3) | 49 (92.5) | | > 0.999 |
| | With | 14 | 7 (9.6) | 7 (9.6) | | | 7 | 3 (5.7) | 4 (7.5) | | |
| T2DM | Without | 131 | 66 (90.4) | 65 (89.0) | 0.074 | 0.785 | 97 | 52 (98.1) | 45 (84.9) | | 0.031 |
| | With | 15 | 7 (9.6) | 8 (11.0) | | | 9 | 1 (1.9) | 8 (15.1) | | |
| HLP | Without | 118 | 60 (82.2) | 58 (79.5) | 0.177 | 0.674 | 91 | 50 (94.3) | 41 (77.4) | 6.290 | 0.012 |
| | With | 28 | 13 (17.8) | 15 (20.5) | | | 15 | 3 (5.7) | 12 (22.6) | | |
| NAFLD | Without | 96 | 55 (75.3) | 41 (56.2) | 5.962 | 0.015 | 65 | 40 (75.5) | 25 (47.2) | 8.949 | 0.003 |
| | With | 50 | 18 (24.7) | 32 (43.8) | | | 41 | 13 (24.5) | 28 (52.8) | | |
| HUA | Without | 98 | 46 (63.0) | 52 (71.2) | | > 0.999 | 78 | 41 (77.4) | 37 (69.8) | 1.603 | 0.205 |
| | With | 48 | 27 (37.0) | 21 (28.8) | | | 28 | 12 (22.6) | 16 (30.2) | | |

Significant postoperative remission of obesity-related comorbidities in all patient groups ($P < 0.001$). SO: Sporadic obesity; FAO: Familial aggregation of obesity; MS: Metabolic syndrome; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; HLP: Hyperlipoidemia; NAFLD: Non-alcoholic fatty liver disease; HUA: Hyperuricemia.

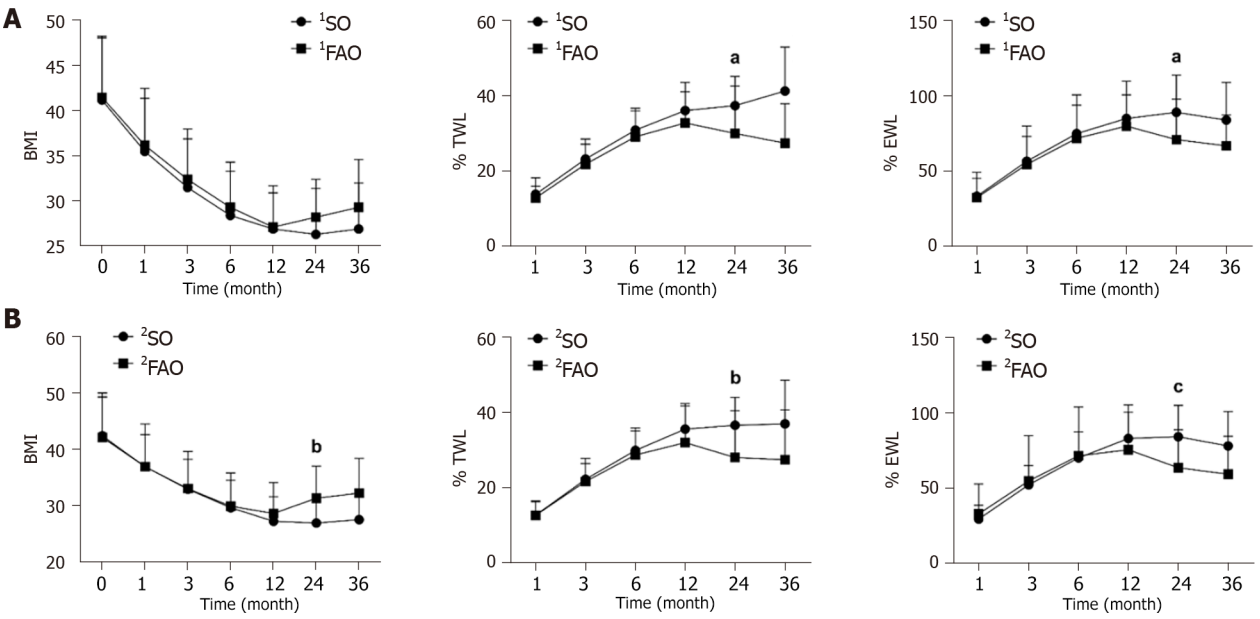


**Figure 1 Line graphs depicting postoperative alterations in body mass index, total weight loss percentage, excess weight loss percentage for patients.** A: In the [1]familial aggregation of obesity (FAO)/[1]sporadic obesity (SO) groups; B: In the [2]FAO/[2]SO groups. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. SO: Sporadic obesity; FAO: Familial aggregation of obesity; %TWL: Total weight loss percentage; %EWL: Excess weight loss percentage; M: Month; BMI: Body mass index.

variations in familial lifestyles across countries and regions. Therefore, initiating a multicenter study involving multiple regions could provide more patients with precise treatment options.

## CONCLUSION

SG can significantly reduce body weight and alleviate obesity-related comorbidities in the majority of patients. Familial aggregation in individuals with obesity impacts the mid-to-long-term weight loss outcomes of SG; affects the alleviation

of T2DM, hyperlipidemia and NAFLD; and leads to a decreased incidence of acid reflux postoperatively. By studying the familial association of obesity, we can gain further insights into the pathogenesis of obesity. Moreover, offering stratified diagnostic and treatment plans for patients with obesity, along with more personalized and targeted health education, can enhance the precision of postoperative prevention and treatment.

## ARTICLE HIGHLIGHTS

### *Research background*

Sleeve gastrectomy (SG) significantly reduces weight and improves obesity-related comorbidities in patients with obesity. However, differences in surgical outcomes between patients with familial aggregation of obesity (FAO) and those with sporadic obesity (SO) have not been elucidated.

### *Research motivation*

To investigate whether FAO influences the surgical outcomes of SG.

### *Research objectives*

To compare preoperative characteristics, postoperative weight loss, resolution of obesity-related comorbidities, and surgical complications between the FAO and SO groups.

### *Research methods*

In this retrospective study, we recruited 193 patients who underwent SG and categorized them into FAO and SO groups based on the presence of obesity in their first-degree relatives. Propensity score matching analysis was used to match the patients at a 1:1 ratio to eliminate confounding factors.

### *Research results*

The baseline data and incidence of obesity-related comorbidities did not significantly differ between FAO patients and SO patients. Two years postsurgery, the FAO group exhibited a lower total weight loss percentage ($P < 0.001$) and excess weight loss percentage ($P < 0.001$) than did the SO group. Significant differences were observed between the two groups in terms of remission rates of type 2 diabetes mellitus (T2DM) ($P = 0.031$), hyperlipidemia ($P = 0.012$), nonalcoholic fatty liver disease ($P = 0.003$), and postoperative reflux occurrence rate ($P = 0.038$).

### *Research conclusions*

Compared to those in the SO group, the FAO patients in the SO group demonstrated slightly weaker medium-term weight loss outcomes; reduced symptoms of T2DM, hyperlipidemia, and nonalcoholic fatty liver disease; and a decreased postoperative reflux rate.

### *Research perspectives*

This study provides a theoretical basis for the treatment, surgical method selection, and postoperative health management of patients with FAO.

## FOOTNOTES

**Author contributions:** Wang ZY designed and performed the research and wrote the paper; Hu SY and Zhong MW designed the research and supervised the report; Qu YF contributed to the analysis; Yu TM and Liu ZL provided clinical advice; and Cheng YG supervised the report.

**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital (Approval No. S447).

**Informed consent statement:** All personal information was encrypted and all data were anonymous. Therefore, informed consent of all study subjects is waived.

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

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**Country/Territory of origin:** China

**ORCID number:** Yu-Gang Cheng 0000-0001-7261-9089; Ming-Wei Zhong 0000-0002-6548-550X; San-Yuan Hu 0000-0002-0546-9778.

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ORIGINAL ARTICLE

Observational Study

# Growth differentiation factor-15 serum concentrations reflect disease severity and anemia in patients with inflammatory bowel disease

Ante Tonkic, Marko Kumric, Ivna Akrapovic Olic, Doris Rusic, Piero Marin Zivkovic, Daniela Supe Domic, Zeljko Sundov, Ivan Males, Josko Bozic

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**Ante Tonkic,** Biology of Neoplasms, University of Split School of Medicine, Split 21000, Croatia

**Marko Kumric, Josko Bozic,** Department of Pathophysiology, University of Split School of Medicine, Split 21000, Croatia

**Ivna Akrapovic Olic, Piero Marin Zivkovic, Zeljko Sundov,** Department of Gastroenterology, University Hospital of Split, Split 21000, Croatia

**Doris Rusic,** Department of Pharmacy, University of Split School of Medicine, Split 21000, Croatia

**Daniela Supe Domic,** Department of Medical Laboratory Diagnostics, University Hospital of Split, Split 21000, Croatia

**Daniela Supe Domic,** Department of Health Studies, University of Split, Split 21000, Croatia

**Zeljko Sundov,** Department of Internal Medicine, University of Split School of Medicine, Split 21000, Croatia

**Ivan Males,** Department of Surgery, University Hospital of Split, Split 21000, Croatia

**Corresponding author:** Josko Bozic, MD, PhD, Associate Professor, Department of Pathophysiology, University of Split School of Medicine, 2 Soltanska, Split 21000, Croatia.
josko.bozic@mefst.hr

## Abstract

### *BACKGROUND*

Population of patients with inflammatory bowel disease (IBD) is burdened by various extraintestinal manifestations which substantially contribute to greater morbidity and mortality. Growth-differentiation factor-15 (GDF-15) is often over-expressed under stress conditions, such as inflammation, malignancies, heart failure, myocardial ischemia, and many others.

### *AIM*

To explore the association between GDF-15 and IBD as serum concentrations of

GDF-15 were shown to be an independent predictor of poor outcomes in multiple diseases. An additional aim was to determine possible associations between GDF-15 and multiple clinical, anthropometric and laboratory parameters in patients with IBD.

***METHODS***

This cross-sectional study included 90 adult patients diagnosed with IBD, encompassing both Crohn's disease (CD) and ulcerative colitis (UC), and 67 healthy age- and sex-matched controls. All patients underwent an extensive workup, including colonoscopy with subsequent histopathological analysis. Disease activity was assessed by two independent gastroenterology consultants specialized in IBD, employing well-established clinical and endoscopic scoring systems. GDF-15 serum concentrations were determined following an overnight fasting, using electrochemiluminescence immunoassay.

***RESULTS***

In patients with IBD, serum GDF-15 concentrations were significantly higher in comparison to the healthy controls [800 (512-1154) pg/mL *vs* 412 (407-424) pg/mL, $P < 0.001$], whereas no difference in GDF-15 was found between patients with CD and UC [807 (554-1451) pg/mL *vs* 790 (509-956) pg/mL, $P = 0.324$]. Moreover, multiple linear regression analysis showed that GDF-15 levels predict CD and UC severity independent of age, sex, and C-reactive protein levels ($P = 0.016$ and $P = 0.049$, respectively). Finally, an association between GDF-15 and indices of anemia was established. Specifically, negative correlations were found between GDF-15 and serum iron levels ($r = -0.248$, $P = 0.021$), as well as GDF-15 and hemoglobin ($r = -0.351$, $P = 0.021$). Accordingly, in comparison to IBD patients with normal hemoglobin levels, GDF-15 serum levels were higher in patients with anemia (1256 (502-2100) pg/mL *vs* 444 (412-795) pg/mL, $P < 0.001$).

***CONCLUSION***

For the first time, we demonstrated that serum concentrations of GDF-15 are elevated in patients with IBD in comparison to healthy controls, and the results imply that GDF-15 might be involved in IBD pathophysiology. Yet, it remains elusive whether GDF-15 could serve as a prognostic indicator in these patients.

**Key Words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Growth-differentiation factor-15; Anemia; Extraintestinal manifestations



**Core Tip:** Serum concentrations of growth-differentiation factor-15 (GDF-15) exhibit a significant elevation in inflammatory bowel disease (IBD) patients compared to healthy controls, irrespective of Crohn's disease or ulcerative colitis diagnosis. GDF-15 levels independently predict disease severity and demonstrate an association with anemia indices, indicating its potential as a biomarker for IBD pathophysiology. Further exploration is nonetheless warranted to determine the prognostic value of GDF-15 in predicting outcomes for patients with IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of chronic inflammatory disorders that affect the gastrointestinal tract, primarily manifesting as Crohn's disease (CD) and ulcerative colitis (UC)[1]. Even though patients with IBD in general exhibit lower prevalence of traditional cardiovascular risk factors, such as hypertension, obesity and dyslipidemia, ample data suggests that the cardiovascular risk by which these patients are burdened exceeds that of the general population[2-6]. Moreover, the population of patients with IBD is burdened by various other extraintestinal manifestations, such as anemia, arthritis, and cancer, which substantially contribute to greater morbidity and mortality[7-10].

Growth-differentiation factor-15 (GDF-15) is a divergent member of the transforming growth factor-β super family[11]. It has been consistently demonstrated that GDF-15 is weakly expressed in all tissue types (except for placenta) under normal physiological states, where it plays a role in cell growth, signal transduction, and apoptosis regulation[11]. On the other hand, GDF-15 is often over-expressed under stress conditions, such as inflammation, malignancies, heart failure, myocardial ischemia and many others[12-15]. In fact, serum concentrations of GDF-15 were shown to predict poor outcomes in conditions with very variegated pathogenesis, for instance colorectal cancer and heart failure[14,15].

Although the exact function of GDF-15 is still not completely understood, it seems that the main function of overexpression in the aforementioned conditions is to maintain cell and tissue homeostasis[16]. Importantly, recent research indicated that serum levels of GDF-15 are associated with subclinical indices of atherosclerosis development in patients with rheumatoid arthritis (RA)[17]. On the other hand, although the data concerning the role of GDF-15 in IBD is limited, recent research indicated a possible association between GDF-15 and indices of iron homeostasis/erythropoiesis in patients with UC[18,19].

Therefore, the aim of the present study was to establish whether serum levels of GDF-15 are higher in patients with IBD in comparison to healthy controls. Moreover, we aimed to determine possible associations between GDF-15 and multiple clinical, anthropometric and laboratory parameters in patients with IBD.

## MATERIALS AND METHODS

### Study design and ethical considerations

The present cross-sectional study was conducted in the Laboratory for Cardiometabolic Research, University of Split School of Medicine and Department of Gastroenterology, University Hospital of Split in the period from January 2022 to January 2023.

The study was conducted in accordance with the ethical principles defined by the Declaration of Helsinki and its amendments, as well as the Good Clinical Practice guidelines from the International Conference on Harmonisation. Ethical approval for the present study was issued by the Ethics Committee of the University Hospital of Split (Class: 500-03/21-01/186; No: 2181-147/01/06/M.S.-21-02; Date: 22 December 2021). Prior to study inclusion, each participant was informed about the goal, procedures, and course of this study and has signed the written informed consent.

### Study population

We consecutively enrolled 90 patients with IBD, 42 of which were diagnosed with UC and 48 with CD. Patients were recruited from the outpatient clinic of the Department of Gastroenterology, University Hospital of Split. In addition, we recruited 67 healthy volunteers with the purpose of forming a control group. The principal inclusion criteria, *i.e.*, IBD diagnosis, was established in the accordance with contemporary guidelines of the European Crohn's and Colitis Organization (ECCO) and the European Society of Gastrointestinal and Abdominal Radiology[20,21]. Specifically, we included patients who fulfilled the following criteria: disease duration of at least one year and stable disease activity in the past three months. The following exclusion criteria were applied: Age under 18 or over 65; established cardiovascular or cerebrovascular disease (heart failure, myocardial infarction, stroke, and peripheral artery disease); history of significant renal, pulmonary, or liver disease; diabetes mellitus; arterial hypertension; autoimmune/chronic inflammatory disorders (other than IBD); history of malignancy; use of corticosteroids in the last 3 months and acute inflammation. The same exclusion criteria applied to the control group. Subjects from the control group were additionally screened for irritable bowel syndrome symptoms according to the Rome IV criteria, as well as other abdominal symptoms suggestive of lactose and/or gluten intolerance[21].

### Clinical assessment and laboratory analysis

Physical examination and relevant items from past medical history were obtained from all participants. Height and weight were measured using calibrated medical scale with integrated altitude meter (Seca, Birmingham, United Kingdom) body mass index was calculated by dividing the value of body mass and the squared value of height. The hip and waist circumferences were measured at standard positions while patients were standing. Waist-to-hip ratio was calculated by dividing the two. Office blood pressure was measured in seating position, following the principles outlined in the contemporary guidelines for management of hypertension[22].

Blood samples for the present analysis were obtained after an overnight fast by an experienced laboratory technician. A maximum of 25 mL of blood was obtained from the cubital vein using a sterile disposable needle. Sampled blood was either immediately analyzed, or aliquoted and stored at -80 °C for subsequent analysis of biomarkers, including GDF-15. GDF-15 serum concentrations were determined using an electrochemiluminescence immunoassay on Cobas e8000 analyzer (Elecsys, Roche Diagnostics). The reported sensitivity for GDF-15 was 400 pg/mL, with a linear range of 400-20000 pg/mL. The inter-assay coefficient of variability was 5%. Fecal calprotectin (FC) concentrations were measured by turbidimetric method (Beckman Coulter AU 680). Reported sensitivity for FC was 15 μg/g, with a linear range of 20-1500 μg/g, and intra-assay coefficient of variability < 20%. The rest of biochemical analyses were conducted by standard laboratory methods by an experienced biochemist. All biochemical analyses in the same certified institutional laboratory, using standard operating procedures, with the biochemist being blinded to the participant's assignment to the IBD or control group.

Disease activity was assessed by two independent gastroenterology consultants specialized in IBD, using well-established clinical and endoscopic scoring systems. In case of score difference between the two, a consensus was made. For CD activity, simple endoscopic score for CD (SES-CD), and CD activity index[23,24]. For patients with UC, we reported Mayo score/disease activity index for UC (Mayo/DAI) and UC endoscopic index of severity (UCEIS)[23,24]. In light of the latest recommendations by ECCO, we used endoscopic index scores (UCEIS and SES-CD, respectively) in assessment of association between GDF-15 and disease severity, whereas other clinical index scores were descriptively reported[20]. Anemia was defined as Hgb < 130 g/L for male, and Hgb < 120 g/L for female patients.

### Statistical analysis

MedCalc version 20.113 (MedCalc Software BV, Ostend, Belgium) and GraphPad Prism version 9.4.1 (GraphPad, La Jolla, CA, United States) were used for statistical data analysis and visual representation of data. Qualitative data was presented as whole number (*n*) and percentage (%), with the Chi-squared ($\chi^2$) test being used for the comparison of categorical variables. Quantitative data was expressed as mean ± SD or median and interquartile range, depending on data distribution. Accordingly, quantitative variables were compared with either Welch's *t*-test or Mann-Whitney *U* test. In light of non-normal distribution of the main parameter of interest, Spearman's rank correlation analysis was used to establish the association between GDF-15 and multiple clinical, laboratory and anthropometric variables. To ascertain that GDF-15 levels differ between IBD and control group independently of the possible confounders, we conducted multiple logistic regression analysis. Covariates for the above-noted analysis were age, sex, C-reactive protein (CRP), low-density lipoprotein (LDL)-cholesterol and albumin levels. Finally, multiple linear regression analysis was used to determine whether GDF-15 serum concentrations might predict disease activity independent of age, sex, and CRP levels. Variance inflation factor was used for detection of multicollinearity in linear regression analysis. Statistical significance was set at $P < 0.05$ for all comparisons.

The sample size was determined based on the analysis of GDF-15 serum levels in a pilot study involving 10 IBD patients and 10 control subjects. Our calculations, with a power of 90% and a type *I* error of 0.05, indicate that 40 subjects are required to detect a significant difference in GDF-15 serum levels.

## RESULTS

In comparison to the control group, patients with IBD were more likely to have a positive family history of IBD ($P = 0.003$), and less likely to have a positive family history of cardiovascular disease (CVD) ($P = 0.041$). Additionally, patients with IBD had higher CRP levels ($P < 0.001$), but lower serum iron ($P = 0.009$), total cholesterol ($P = 0.008$), LDL-cholesterol ($P < 0.001$), and albumin levels ($P < 0.001$) compared to the control group. No significant differences were noted in other variables of interest. The baseline characteristics of patients are summarized in Table 1.

Compared to patients with UC, those with CD exhibited a higher prevalence of smoking ($P < 0.001$), extraintestinal manifestations ($P < 0.001$), and a greater likelihood of undergoing IBD-related surgery previously ($P < 0.001$). Furthermore, CD patients showed higher FC levels ($P = 0.009$) but lower levels of albumin ($P < 0.001$), total cholesterol ($P = 0.005$), LDL-cholesterol ($P < 0.001$), and high-density lipoprotein-cholesterol ($P = 0.045$). A comprehensive comparison of relevant basic characteristics, laboratory parameters, and disease features between patients with CD and UC is delineated in Table 2.

In comparison to the healthy age and sex-matched control group, GDF-15 serum concentrations were significantly higher in patients with IBD ($P < 0.001$) (Table 3). Additionally, multiple logistic regression analysis revealed that GDF-15 serum levels predict the presence of IBD independently of age, sex, CRP, albumin, and LDL serum levels (odds ratio: 1.17, 95% confidence interval: 1.05-1.19, $P < 0.001$). Serum concentrations of GDF-15 did not exhibit a significant difference between patients with CD and UC ($P = 0.324$) (Table 3).

Significant correlations were noted between GDF-15 serum levels and IBD endoscopic disease activity indices. Specifically, GDF-15 serum levels in patients with CD were found to correlate with SES-CD ($r = 0.405$, $P = 0.004$) (Figure 1A), whereas in UC, a positive correlation was noted between GDF-15 levels and UCEIS ($r = 0.391$, $P = 0.010$) (Figure 1B). Moderate positive correlation was also found between CRP and GDF-15 in the total studied population ($r = 0.406$, $P < 0.001$) (Figure 1C). Nonetheless, it is worth noting that multiple linear regression analysis showed that GDF-15 levels predict CD and UC severity independent of age, sex, and CRP levels ($P = 0.016$ and $P = 0.049$, respectively).

On the other hand, negative correlations were found between GDF-15 and serum iron levels ($r = -0.248$, $P = 0.021$), as well as GDF-15 and hemoglobin ($r = -0.351$, $P = 0.021$) (Figure 1D and E). Accordingly, in comparison to IBD patients with normal hemoglobin levels, GDF-15 serum levels were higher in patients with anemia [1256 (502-2100) pg/mL *vs* 444 (412-795) pg/mL, $P < 0.001$] (Figure 1F). However, when analyses for UC and CD were conducted separately, the difference in GDF-15 with respect to the presence of anemia [859.5 (502.0-2464.0) pg/mL *vs* 787.0 (531.0-940.3) pg/mL, $P = 0.396$], as well as correlations with the aforementioned variables (serum iron and hemoglobin), were lost ($r = -0.147$, $P = 0.359$ and $r = 0.003$, $P = 0.984$, respectively).

Finally, we performed a correlation analysis between serum concentrations of GDF-15 and multiple anthropometric and laboratory variables. Significant correlations with serum GDF-15 were observed for albumins ($r = -0.338$), total cholesterol ($r = -0.196$) and LDL-cholesterol ($r = -0.216$). The results of the analysis are presented in Table 4.

## DISCUSSION

To the best of our knowledge, this is the first study in which serum concentrations of GDF-15 were compared between patients with IBD and healthy age and sex-matched controls, and the only report in which the association between GDF-15 and IBD severity was explored.

There is a paucity of data concerning the role of GDF-15 in IBD, especially in human subjects. In a recent study, Yamamoto *et al*[18] reported that GDF-15 serum levels are significantly higher in CD patients with low skeletal muscle mass index (SMI) in comparison to high SMI, even after adjusting for possible confounders. Accordingly, negative correlation was established between GDF-15 and SMI. A possible explanation of this association is that GDF-15 may

**Table 1 Baseline characteristics of the studied population**

| Parameter | Control group (*n* = 67) | IBD group (*n* = 90) | *P* value[1] |
|---|---|---|---|
| Age, yr | 38.5 ± 12.3 | 41.2 ± 15.8 | 0.195 |
| Male sex, *n* (%) | 43 (64.2) | 53 (58.9) | 0.503 |
| Body mass index, kg/m$^2$ | 24.7 ± 2.8 | 23.8 ± 4.2 | 0.070 |
| Waist-to-hip ratio | 0.91 ± 0.36 | 0.87 ± 0.08 | 0.368 |
| Hypertension, *n* (%) | 1 (1.5) | 6 (6.7) | 0.122 |
| Dyslipidemia, *n* (%) | 2 (3.0) | 7 (7.8) | 0.203 |
| Family history of IBD, *n* (%) | 3 (4.5) | 16 (17.8) | 0.012 |
| Family history of CRC, *n* (%) | 9 (13.4) | 17 (18.9) | 0.365 |
| Family history of CVD, *n* (%) | 31 (46.3) | 29 (32.2) | 0.074 |
| Smoking, *n* (%) | 12 (18.5) | 17 (18.9) | 0.947 |
| C-reactive protein, mg/L | 0.7 (0.4-1.6) | 1.6 (0.7-3.8) | < 0.001 |
| Serum iron, μmol/L | 18.1 ± 6.7 | 15.2 ± 7.8 | 0.009 |
| Albumins, g/L | 43.9 ± 2.5 | 40.4 ± 4.9 | < 0.001 |
| Serum urate levels, mmol/L | 296.1 ± 76.0 | 275.5 ± 70.9 | 0.064 |
| Fasting blood glucose, mmol/L | 5.1 ± 0.7 | 5.2 ± 1.6 | 0.691 |
| Total cholesterol, mmol/L | 5.2 ± 1.2 | 4.7 ± 1.3 | 0.008 |
| LDL-C, mmol/L | 3.3 ± 1.1 | 2.7 ± 1.1 | < 0.001 |
| HDL-C, mmol/L | 1.4 ± 0.3 | 1.4 ± 0.4 | 0.409 |
| Triglycerides, mmol/L | 1.2 ± 0.6 | 1.4 ± 1.1 | 0.158 |

[1]Welch's *t*-test, Mann-Whitney *U* test or chi squared test, as appropriate.

Data presented as mean ± SD, median (IQR) or *n* (%). CRC: Colorectal carcinoma; CVD: Cardiovascular disease; HDL-C: High-density lipoprotein cholesterol; IBD: Inflammatory bowel disease; LDL-C: Low-density lipoprotein cholesterol.

promote muscle wasting. Aside from the direct catabolic effects, it has been reported that the binding of GDF-15 to the receptors in the brainstem may lead to the loss of appetite and concurrent weight loss[25-30]. Although Yamamoto *et al* [18] did not have a control group, GDF-15 levels were similar to that of our CD population, and significantly higher than in healthy controls from our study or from previous reports[31,32]. In contrast to our results, a recent study failed to demonstrate a difference in GDF-15 serum levels between patients with UC and healthy controls[19]. The probable cause of the disparity is the fact that our study included significantly higher proportion of severe UC cases (approximately 30% *vs* approximately 10%). Since patients in the above-noted study were appropriately matched with controls, and as control subjects seem to be concordant with ours in terms of age and sex distribution, another possible explanation of conflicting results is ethnicity difference. Specifically, all our patients were of European ancestry, in contrast to the study by Ramasamy *et al*[19] that included Indian population exclusively. Although a conclusive answer on why the GDF-15 levels is elevated in IBD regardless of the CRP levels cannot be reached with the current study design, in line with the available data in other autoimmune disorders, we hypothesized that the elevated GDF-15 reflect its protective role in IBD. For instance, preclinical studies demonstrated that mice deficient in GDF-15 exhibited a more pronounced systemic inflammatory response, marked by increased levels of IL-6, IL-12, tumour necrosis factor alpha, and interferon-gamma in the serum, as well as enhanced local inflammatory response characterized by increased T-cell infiltration and upregulation of CXCR3 in a model of membrane glomerulonephritis[33].

Positive correlation between GDF-15 and endoscopic indices of CD and UC is concordant with previous reports in other chronic diseases[34]. In patients with RA, multiple authors demonstrated a positive correlation between the disease severity and the GDF-15 serum levels[17,35]. Brown *et al*[35] even demonstrated that applying GDF-15 in algorithms may aid in predicting response to hematopoietic stem cell transplantation, the presence of severe form, and joint erosions in RA. A preliminary report in patients with spondyloarthritis is in line with the data from RA studies, as a pilot study showed a moderate correlation between GDF-15 and multiple indices of spondyloarthritis severity[36]. Of note, in idiopathic inflammatory myopathy, GDF-15 serum levels correlated with the extent of myocardial injury[37]. Unfortunately, in previously conducted studies that measured GDF-15 serum levels in IBD patients, an association between disease severity and GDF-15 was not explored.

As anemia is a well-established extraintestinal manifestation of IBD, the presence of higher GDF-15 levels in anemic patients, as well as negative correlations of GDF-15 with both hemoglobin and serum iron, deserve particular attention. However, inferring about causative relation between any biomarker and anemia in IBD is challenging owing to its dual

**Table 2 Basic anthropometric and disease characteristics of ulcerative colitis and Crohn's disease group**

| Parameter | Crohn's disease (*n* = 48) | Ulcerative colitis (*n* = 42) | *P* value[1] |
|---|---|---|---|
| Basic characteristics | | | |
| Age, yr | 40.7 ± 16.0 | 42.7 ± 15.9 | 0.567 |
| Male sex, *n* (%) | 31 (64.6) | 22 (52.4) | 0.243 |
| Body mass index, kg/m$^2$ | 23.2 ± 3.6 | 23.9 ± 4.9 | 0.459 |
| Waist-to-hip ratio | 0.86 ± 0.08 | 0.86 ± 0.08 | 0.495 |
| Hypertension, *n* (%) | 2 (4.2) | 4 (9.5) | 0.312 |
| Dyslipidemia, *n* (%) | 3 (6.2) | 4 (9.5) | 0.565 |
| Family history of IBD, *n* (%) | 10 (20.8) | 6 (14.3) | 0.420 |
| Family history of CRC, *n* (%) | 10 (20.8) | 7 (16.7) | 0.616 |
| Family history of CVD, *n* (%) | 12 (28.6) | 17 (35.4) | 0.491 |
| Smoking, *n* (%) | 16 (33.3) | 1 (2.4) | < 0.001 |
| Laboratory parameters | | | |
| C-reactive protein, mg/L | 1.9 (0.6-7.6) | 1.4 (0.8-2.2) | 0.153 |
| Serum iron, μmol/L | 14.6 ± 7.6 | 16.5 ± 7.9 | 0.247 |
| Albumins, g/L | 39.5 ± 5.5 | 41.6 ± 3.6 | 0.018 |
| Fasting blood glucose, mmol/L | 4.9 ± 0.8 | 5.6 ± 2.5 | 0.100 |
| Total cholesterol, mmol/L | 4.4 ± 1.2 | 5.1 ± 1.3 | 0.005 |
| LDL-C, mmol/L | 2.3 ± 0.9 | 3.1 ± 1.1 | < 0.001 |
| HDL-C, mmol/L | 1.3 ± 0.4 | 1.4 ± 0.4 | 0.045 |
| Triglycerides, mmol/L | 1.7 ± 1.5 | 1.2 ± 0.7 | 0.079 |
| Disease characteristics | | | |
| Disease duration, yr | 7 (3-14) | 9 (5-13) | 0.397 |
| IBD-related surgery, *n* (%) | 20 (41.7) | 0 (0.0) | < 0.001 |
| ExtraintestiN/Al manifestations, *n* (%) | 27 (56.3) | 7 (16.7) | < 0.001 |
| SES-CD | 10 (5-13) | N/A | N/A |
| CDAI | 55 (34-84) | N/A | N/A |
| UCEIS | N/A | 5.0 (1.5-6.5) | |
| Mayo/DAI | N/A | 3 (2-5) | N/A |
| Fecal calprotectin, μg/g | 232 (80-589) | 85 (10-246) | 0.009 |
| Therapy, *n* (%) | | | |
| Aminosalicylates | 22 (45.8) | 23 (54.8) | 0.421 |
| DMARDs | 18 (37.5) | 17 (40.5) | 0.826 |
| MonocloN/Al antibodies | 36 (75.0) | 32 (76.2) | 0.896 |

[1]Welch's *t*-test, Mann-Whitney *U* test or chi squared test, as appropriate.

Data presented as mean ± SD, median (IQR) or *n* (%). CDAI: Crohn's disease activity index; CRC: Colorectal carcinoma; CVD: Cardiovascular disease; DMARD: Disease-modifying anti-rheumatic drugs; HDL-C: High-density lipoprotein cholesterol; IBD: Inflammatory bowel disease; LDL-C: Low-density lipoprotein cholesterol; Mayo/DAI: Mayo score/disease activity index; SES-CD: Simple endoscopic score for Crohn's disease; UCEIS: Ulcerative colitis endoscopic index of severity; N/A: Not applicable.

pathophysiology: Iron deficiency anemia develops as a result of chronic blood loss in IBD, whereas chronic inflammation underlies the development of anemia of chronic disease[38]. Previous research has indicated that GDF-15 levels correlate with anemia severity in patients with cancer, and that GDF-15 is in fact a negative regulator of hepcidin, a central regulator of iron homeostasis[39-41]. However, a recent study failed to demonstrate a correlation between GDF-15 and hepcidin in patients with UC[19]. In addition, the authors did not find any difference in the GDF-15 serum levels between

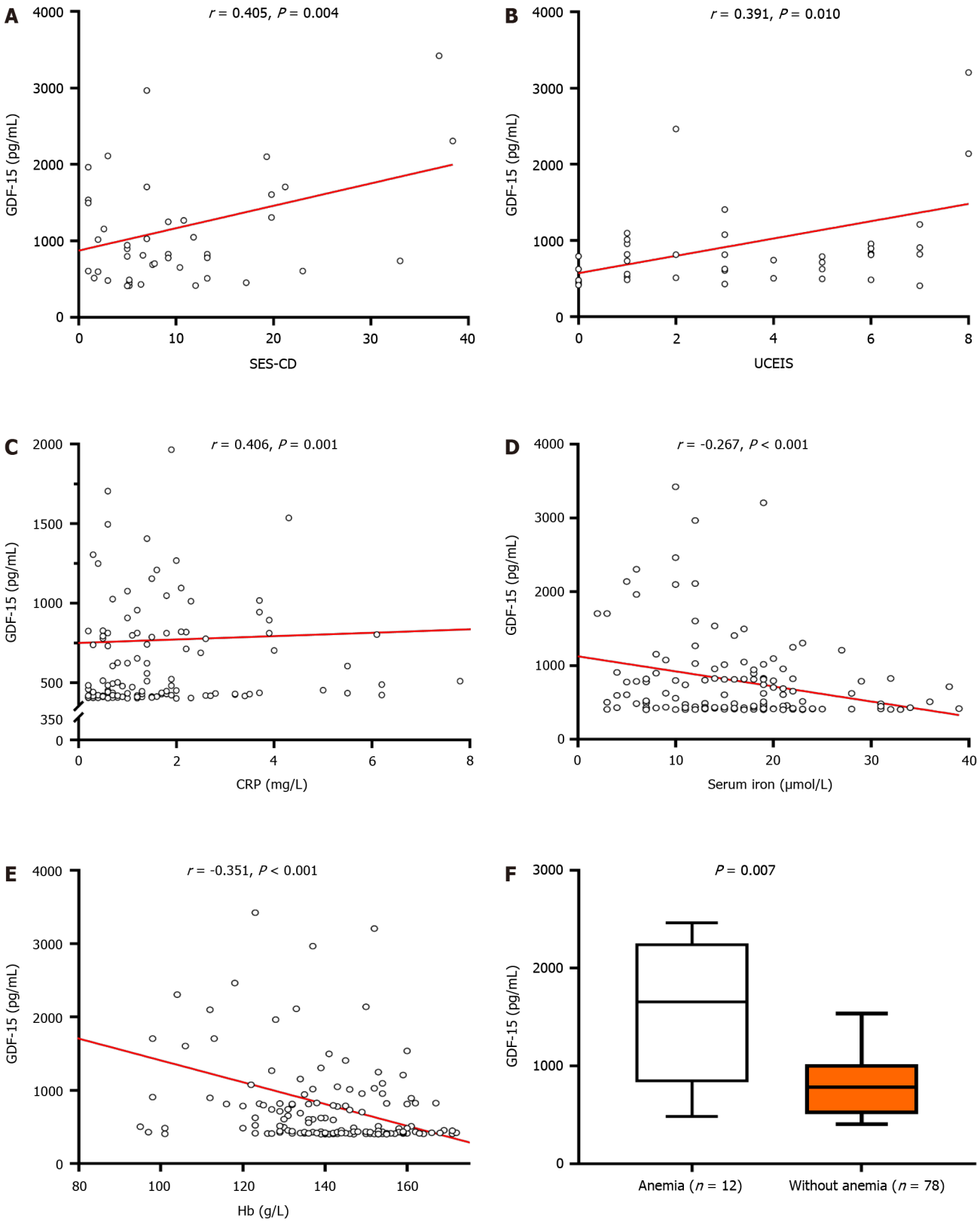


**Figure 1 Correlation and comparison.** A: Correlation between growth-differentiation factor-15 (GDF-15) serum levels and simple endoscopic score for Crohn's disease (CD) in patients with CD (Spearman's rank correlation coefficient); B: Correlations between GDF-15 serum levels and ulcerative colitis endoscopic index of severity in patients with ulcerative colitis (Spearman's rank correlation coefficient); C: Correlation between GDF-15 and C-reactive protein in the total studied population (Spearman's rank correlation coefficient); D and E: Correlation between GDF-15 and indicators of anemia in the total studied population: Serum iron levels (D) and hemoglobin (E) (Spearman's rank correlation coefficient); F: Comparison of serum GDF-15 concentrations with respect to the presence of anemia in patients with inflammatory bowel disease (Mann-Whitney *U* test). GDF-15: Growth-differentiation factor-15; SES-CD: Simple endoscopic score for Crohn's disease; UCEIS: Ulcerative colitis endoscopic index of severity; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; Hb: Hemoglobin; CRP: C-reactive protein.

**Table 3 Comparison of serum growth-differentiation factor-15 concentrations between the study groups**

| Parameter | Study groups | | *P* value[1] |
|---|---|---|---|
| GDF-15, pg/mL | Control group ($n$ = 67) | IBD group ($n$ = 90) | |
| | 412 (407-424) | 800 (512-1154) | < 0.001 |
| | Crohn's disease ($n$ = 48) | Ulcerative colitis ($n$ = 42) | |
| | 807 (554-1451) | 790 (509-956) | 0.324 |

[1]Mann-Whitney *U* test. Data are presented as median (interquartile range).
GDF-15: Growth-differentiation factor-15; IBD: Inflammatory bowel disease.

**Table 4 Correlation analysis between growth-differentiation factor-15 and various anthropometric and laboratory variables**

| Parameter | *r*-correlation coefficient[1] | *P* value |
|---|---|---|
| Age, yr | 0.117 | 0.145 |
| Albumins | -0.338 | < 0.001 |
| Fecal calprotectin[2] | 0.143 | 0.178 |
| Fasting glucose | 0.021 | 0.796 |
| Total cholesterol | -0.196 | 0.014 |
| LDL-cholesterol | -0.216 | 0.007 |
| Waist-to-hip ratio | -0.044 | 0.584 |

[1]Spearman's rank correlation coefficient.
[2]Measured only in patients with inflammatory bowel disease.
IBD: Inflammatory bowel disease; LDL-cholesterol: Low-density lipoprotein cholesterol.

anemic and non-anemic UC patients, which is in line with our sub-analysis on UC patients, but not on the overall IBD population. The authors argued that lack of change reflects insufficient anemia severity needed to induce GDF-15 secretion, which is also consistent with our data, *i.e.*, negative correlation between GDF-15 and hemoglobin levels. Significant correlations in CD but not UC, are challenging to interpret in the absence of data for comparison and limited sample size, but since hemoglobin, serum iron and GDF-15 levels in our study were similar between UC and CD patients, we argue that there is a possibility that pathophysiological mechanisms underlying anemia in our UC and CD population may not be completely concordant.

Although GDF-15 has been shown to be a very successful prognostic indicator in multiple diseases, especially those of cardiovascular origin, current study design prevents us from making inferences about prognostic value of GDF-15 in IBD population. Yet, in light of the fact that serum concentrations of GDF-15 were associated with disease severity and anemia in patients with IBD, both of which could potentially contribute to morbidity and mortality, GDF-15 might serve as a predictor of poor outcomes beyond traditional risk factors in IBD population, providing a strong basis to explore such association in future studies. Of important note, the pathophysiological processes that underlie the relationship between GDF-15 and CVD are still elusive, rendering the interpretation difficult[33]. In fact, as GDF-15 appears to produce both beneficial and adverse effects depending on the microenvironment, it remains unclear whether GDF-15 offers protective or detrimental role.

The present study has several limitations. Cross-sectional design prevents us from establishing causality. Furthermore, relapsing, and remitting nature of IBD alongside non-constant secretion of GDF-15 further impedes the establishment of causality by single point measurement. Nonetheless, it is worth noting that patients were equally distributed with respect to disease activity in both UC and CD. The study might benefit from concurrent measurement of serum hepcidin and soluble transferrin receptor concentrations. Finally, although sample size was somewhat limited, we aimed to create IBD and control groups devoid of any other pathologies that might violate the assumptions about the role of GDF-15 in IBD.

## CONCLUSION

For the first time, we demonstrated that serum concentrations of GDF-15 are elevated in patients with IBD in comparison to age and sex-matched healthy controls independently of the factors that might affect GDF-15 levels. Moreover, in patients with both CD and UC, a positive correlation was found between GDF-15 and endoscopic disease activity indices, whereas no significant difference in GDF-15 serum levels was found between CD and UC groups. In addition, an

association between the serum iron and hematological parameters of anemia with GDF-15 serum levels was established in patients with CD. Overall, although the present results implicate that GDF-15 might be involved in pathophysiology of IBD and its extraintestinal manifestations, currently there is insufficient data to establish whether serum GDF-15 levels might predict outcomes in patients with IBD. Exploring molecular pathways associating GDF-15 and IBD, and adequately powered prospective studies that assess crude outcomes represent the two crucial tools required to put these premises to the test. Finally, given the well-established role of GDF-15 in predicting cardiovascular outcomes, future research needs to elucidate whether GDF-15 explains paradoxically worse cardiovascular outcomes in patients with IBD.

## ARTICLE HIGHLIGHTS

### *Research background*

Although commonly perceived as disease of the gastrointestinal system, inflammatory bowel disease can affect other organ systems, including cardiovascular, potentially leading to increased morbidity and mortality. Growth-differentiation factor-15 (GDF-15) is often over-expressed in stress conditions, including inflammation, malignancies, heart failure, and myocardial ischemia. In fact, elevated serum concentrations of GDF-15 have been linked to poor outcomes in conditions with diverse pathogenesis, such as colorectal cancer and heart failure.

### *Research motivation*

As serum concentrations of GDF-15 were shown to be an independent predictor of poor outcomes in diverse ailments, we aimed to explore whether such association is present in the setting of inflammatory bowel disease (IBD) and its consequences. Establishing the role of GDF-15 in IBD might be relevant since poor long-term outcomes in IBD population are currently not fully elucidated.

### *Research objectives*

To establish whether serum levels of GDF-15 in patients with IBD are different then in the healthy controls. Furthermore, we aimed to establish whether GDF-15 level correlate with disease severity, thus providing a rationale for the future assessment of its prognostic role in IBD. Finally, we investigated if association between indices of anemia and GDF-15 serum levels exists in this population.

### *Research methods*

In this cross-sectional study, patients with IBD and healthy age- and sex-matched participants underwent an extensive diagnostic workup. IBD group also underwent colonoscopy with subsequent histopathological analysis, and the disease activity was assessed using well-established clinical and endoscopic scoring systems. GDF-15 serum concentrations were determined using electrochemiluminescence immunoassay.

### *Research results*

The principal findings of the present study reveal significantly elevated levels of GDF-15 in patients with IBD compared to the control group, and these levels increase with greater disease severity. Since no similar data have been previously published, the reasons behind this observation remain elusive. Nevertheless, considering the independent association between GDF-15 and indices of anemia, it is plausible that pathophysiological changes in anemia and iron metabolism might, to some extent, explain the observed difference.

### *Research conclusions*

This study marks the first demonstration of significantly elevated serum concentrations of GDF-15 in patients with IBD. While mechanistic studies and prospective trials are essential for firm conclusions, these preliminary findings suggest that exploring the role of GDF-15 as a biomarker in IBD could be worthwhile.

### *Research perspectives*

Future research should delve into the prognostic role of GDF-15, with a specific focus on its relationship with disease severity. Furthermore, investigating the mechanisms underlying these preliminary results will contribute to a deeper understanding of the role of GDF-15 in IBD.

## FOOTNOTES

**Author contributions:** Tonkic A participated in conceptualization, methodology, investigation, formal analysis, and original draft preparation; Kumric M participated in visualization, investigation, formal analysis, and original draft preparation; Akrapovic Olic I participated in visualization, investigation, formal analysis, and original draft preparation; Rusic D participated in visualization, investigation, formal analysis, and reviewing and editing of the manuscript; Zivkovic PM participated in visualization, investigation, formal analysis, and original draft preparation; Supe Domic D participated in visualization, investigation, formal analysis, and original draft preparation; Sundov Z participated in visualization, investigation, formal analysis and reviewing, and editing of the manuscript; Males I participated in visualization, investigation, formal analysis, and original draft preparation; Bozic J participated in conceptualization, funding acquisition, resources, project administration, and reviewing and editing of the manuscript; and all authors

contributed to the final draft of the manuscript, read and agreed to the published version of the manuscript.

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

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**Country/Territory of origin:** Croatia

**ORCID number:** Marko Kumric 0000-0002-9696-3359; Doris Rusic 0000-0002-7018-4947; Piero Marin Zivkovic 0000-0001-5649-698X; Josko Bozic 0000-0003-1634-0635.

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ORIGINAL ARTICLE

Basic Study

# Inhibition of hepatitis B virus *via* selective apoptosis modulation by Chinese patent medicine Liuweiwuling Tablet

Fei-Lin Ge, Yan Yang, Lan-Lan Si, Yuan-Hua Li, Meng-Zhen Cao, Jun Wang, Zhao-Fang Bai, Zhi-Gang Ren, Xiao-He Xiao, Yan Liu

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**Fei-Lin Ge,** Department of Chinese Medicine, State Key Laboratory of Antiviral Drugs, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

**Fei-Lin Ge, Lan-Lan Si, Yuan-Hua Li, Meng-Zhen Cao, Zhao-Fang Bai, Xiao-He Xiao, Yan Liu,** The Fifth Medical Center of Chinese PLA General Hospital, Beijing 100039, China

**Yan Yang,** College of Traditional Chinese Medicine, Chongqing Medical University, Chongqing 400010, China

**Jun Wang,** Beijing Key Laboratory of Emerging Infectious Diseases, Peking University Ditan Teaching Hospital, Beijing 100015, China

**Zhi-Gang Ren,** Department of Infectious Diseases, State Key Laboratory of Antiviral Drugs, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

**Corresponding author:** Yan Liu, PhD, The Fifth Medical Center of Chinese PLA General Hospital, No. 100 Xisihuan Middle Road, Beijing 100039, China. liuyan5360@163.com

## Abstract

### *BACKGROUND*

Liuweiwuling Tablet (LWWL) is a Chinese patent medicine approved for the treatment of chronic inflammation caused by hepatitis B virus (HBV) infection. Previous studies have indicated an anti-HBV effect of LWWL, specifically in terms of antigen inhibition, but the underlying mechanism remains unclear.

### *AIM*

To investigate the potential mechanism of action of LWWL against HBV.

### *METHODS*

*In vitro* experiments utilized three HBV-replicating and three non-HBV-replicating cell lines. The *in vivo* experiment involved a hydrodynamic injection-mediated mouse model with HBV replication. Transcriptomics and metabolomics were used to investigate the underlying mechanisms of action of LWWL.

### *RESULTS*

In HepG2.1403F cells, LWWL (0.8 mg/mL) exhibited inhibitory effects on HBV DNA, hepatitis B surface antigen and pregenomic RNA (pgRNA) at rates of 51.36%, 24.74% and 50.74%, respectively. The inhibition rates of LWWL (0.8

mg/mL) on pgRNA/covalently closed circular DNA in HepG2.1403F, HepG2.2.15 and HepG2.A64 cells were 47.78%, 39.51% and 46.74%, respectively. Integration of transcriptomics and metabolomics showed that the anti-HBV effect of LWWL was primarily linked to pathways related to apoptosis (PI3K-AKT, CASP8-CASP3 and P53 pathways). Apoptosis flow analysis revealed that the apoptosis rate in the LWWL-treated group was significantly higher than in the control group (CG) among HBV-replicating cell lines, including HepG2.2.15 (2.92% ± 1.01% *vs* 6.68% ± 2.04%, $P < 0.05$), HepG2.A64 (4.89% ± 1.28% *vs* 8.52% ± 0.50%, $P < 0.05$) and HepG2.1403F (3.76% ± 1.40% *vs* 7.57% ± 1.35%, $P < 0.05$) (CG *vs* LWWL-treated group). However, there were no significant differences in apoptosis rates between the non-HBV-replicating HepG2 cells (5.04% ± 0.74% *vs* 5.51% ± 1.57%, $P > 0.05$), L02 cells (5.49% ± 0.80% *vs* 5.48% ± 1.01%, $P > 0.05$) and LX2 cells (6.29% ± 1.54% *vs* 6.29% ± 0.88%, $P > 0.05$). TUNEL staining revealed a significantly higher apoptosis rate in the LWWL-treated group than in the CG in the HBV-replicating mouse model, while no noticeable difference in apoptosis rates between the two groups was observed in the non-HBV-replicating mouse model.

*CONCLUSION*

Preliminary results suggest that LWWL exerts a potent inhibitory effect on wild-type and drug-resistant HBV, potentially involving selective regulation of apoptosis. These findings offer novel insights into the anti-HBV activities of LWWL and present a novel mechanism for the development of anti-HBV medications.

**Key Words:** Hepatitis B virus; Chinese patent medicine; Antiviral activity; Antiviral mechanism; Selective apoptosis



**Core Tip:** Liuweiwuling Tablet (LWWL) exerts a potent inhibitory effect on both wild-type and drug-resistant hepatitis B virus (HBV) models. This effect appears to hinge on selective apoptosis regulation-an unprecedented discovery in the realm of anti-HBV drug mechanisms. These revelatory findings not only deepen our understanding of LWWL's anti-HBV properties but also offer an innovative mechanism to inspire the development of new anti-HBV drugs.

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## INTRODUCTION

Hepatitis B virus (HBV) infection can lead to chronic hepatitis B (CHB) and increase the risk of liver cirrhosis and hepatocellular carcinoma. The estimated 257 million people annually who are positive for hepatitis B surface antigen (HBsAg) suffer > 887000 deaths[1]. China has the highest number of CHB patients globally (70 million), resulting in an annual direct medical burden of up to ¥600 billion[2,3]. Consequently, CHB remains one of the most critical global public health concerns. Interferon and nucleoside/nucleotide analogs (NAs), two types of anti-HBV medications, have obtained licenses for treating HBV-related diseases. Currently, six NAs have been authorized in China for CHB treatment. Despite the considerable benefits these antiviral drugs have offered patients, the challenge of eradicating HBV from individuals with chronic infection using current medication remains substantial. Additional factors affecting treatment success include adverse drug responses and HBV drug resistance. Substantial endeavors have been made to develop more efficacious drugs and therapies[4,5], aiming to facilitate functional cure for CHB.

Liuweiwuling Tablet (LWWL) (approval number: Z20060238) has been granted approval by the China National Medical Products Administration. This is included in the national health insurance program and exhibits notable anti-inflammatory properties[6,7]. In our earlier research using the wild-type HBV model, we observed an anti-HBV effect of LWWL. Particularly notable is the suppression of HBV antigen, which compensates for the absence of NAs[8]. Our meta-analysis of its clinical effects indicates that combination of LWWL with NAs increases the undetectability of HBV DNA and hepatitis B e antigen (HBeAg) when compared with NAs alone[9]. However, the lack of clarity of its antiviral mechanism restricts its potential therapeutic application. The objective of this study was to elucidate the antiviral activity of LWWL. This was achieved by investigating its potential mechanism through the integration of transcriptomics and metabolomics. Additionally, a more comprehensive evaluation of the anti-HBV effect of LWWL was conducted within a drug-resistant HBV model.

## MATERIALS AND METHODS

### *Evaluating anti-HBV efficacy of LWWL in cell models*

We utilized three HBV-replicating cell lines: HepG2.2.15 (wild-type), HepG2.A64 (entecavir-resistant) and HepG2.1403F (multidrug-resistant). Cytotoxicity of LWWL (Shibo Jindu, Zibo, China) was assessed using the Cell Counting Kit-8 (Dojindo Laboratories, Kyushu, Japan). The molecular and cellular experiments were conducted within the biosafety level-2 Laboratory at the Fifth Medical Center of the Chinese PLA General Hospital. All procedures were executed in full adherence to the established protocols. The 50% cytotoxicity concentration ($CC_{50}$) for cultured cells was calculated.

HepG2.1403F cells were seeded in 48-well plates at $2 \times 10^4$ per well with various drug concentrations [0, 0.1, 0.2, 0.4 or 0.8 mg/mL LWWL, or 0, 0.2, 2, 20 or 200 μmol/L tenofovir disoproxil fumarate (TDF)]. During 120-h cultivation, cell supernatants were harvested to measure HBsAg and HBeAg levels using Enzyme-linked immunosorbent assay kits (Wantai Biological Pharmacy Enterprise Co. Ltd., Beijing, China). Concurrently, cells were harvested to determine HBV DNA levels *via* quantitative PCR (qPCR) assays as described previously[10,11]. The 50% inhibitory concentration ($IC_{50}$) and selectivity index (ratio between $CC_{50}$ and $IC_{50}$) were calculated.

Three HBV-replicating cell lines were individually seeded in 48-well plates at $2 \times 10^4$ per well in triplicate with or without 0.8 mg/mL LWWL, or 200 μmol/L. During 120-h cultivation, cell supernatants were collected for HBV pregenomic RNA (pgRNA) analysis using quantitative reverse transcription PCR. Additionally, adherent cells were harvested to quantify HBV covalently closed circular DNA (cccDNA) *via* qPCR, as previously outlined[12,13].

### *Evaluating anti-HBV efficacy of LWWL in mouse models*

Male SPF C57BL/6 mice, aged 6-8 wk, weight 18-20 g, were purchased from Sibeifu, Beijing (animal license No. SCXK (Jing) 2019-0016). The animals were kept in the SPF laboratory of the Fifth Medical Center of the Chinese PLA General Hospital and were allowed to eat and drink freely except for fasting tests. The room was kept at 22 ± 2 °C in a 12-h light/dark cycle, with 50% humidity. After 1 wk of adaptive feeding, mice were subjected to follow-up experiments. pAAV-HBV1.2 HBV plasmid (20 mg) was injected into mice through the tail vein within 5-8 s using a high-pressure hydrodynamic method to establish the HBV-replicating mouse model. The mice were divided into four groups ($n$ = 6) and received intraperitoneal treatments once daily for 4 wk. Group 1 served as the control and received normal saline (NS). Group 2 was treated with a low dose of LWWL (1 g/kg/d); group 3 received a high dose of LWWL (2 g/kg/d); and group 4 was treated with TDF (63 mg/kg/d). The levels of HBV antigens and DNA were measured. The animal study was conducted at the Animal Laboratory Center, Fifth Medical Center of the Chinese PLA General Hospital, following strict adherence to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol received approval from the Committee on the Ethics of Animal Experiments at the Fifth Medical Center of the Chinese PLA General Hospital (Permit number: IACUC-2021-0009).

### *Metabolomics analysis of LWWL in HBV-replicating mouse models*

The serum samples were obtained from section "Evaluating anti-HBV efficacy of LWWL in mouse models" of the materials and methods. Then, 200 μL serum was extracted, followed by addition of 600 μL methanol. After 30 s of vortex oscillation, the mixture was centrifuged at 13000 rpm for 10 min, resulting in collection of 450 μL superserum. The superserum was subjected to extraction and concentrated to dryness through a vacuum centrifugal concentrator. The residue was dissolved using 200 μL methanol, followed by 30 s of vortexing. Supernatant samples were obtained by centrifugation at 15000 rpm for 10 min. We used the ACQUITY UPLC HSS T3 chromatographic column (100 mm × 2.1 mm, 1.8 μm). The column temperature was set at 45 °C. The mobile phase comprised A-water (with 0.1% formic acid) and B-acetonitrile (with 0.1% formic acid). The flow rate was 0.35 mL/min, and the injection volume was 2 μL. The elution gradient is detailed in Supplementary Table 1. Regarding the mass spectrum conditions, the ion source used was electrospray ionization, and both positive and negative ion scanning modes were utilized to gather signals for the sample quality spectrum (Supplementary Table 2).

Chromatographic peaks were imported into MetaboAnalyst 3.0, followed by standardized processing based on peak area. The standardized data were analyzed using SIMCA-P-13.0, with principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Potential biomarkers were identified by analyzing the two OPLS-DA groups, setting a threshold of $P < 0.05$ and variable importance in the projection (VIP) > 1. Potential biomarkers underwent biological function annotation and metabolic pathway enrichment analysis through Mass Hunter PCDL Manager software, MetaboAnalyst website, Kyoto Encyclopedia of Genes and Genomes (KEGG), and various other metabolomics and biological databases. The key differential metabolites were selected for receiver operating characteristic curve analysis, aligning with the research objective and biological functions. The area under the curve (AUC) was calculated to determine the sensitivity and specificity of these differential metabolites. A differential metabolite with an AUC closer to 1 indicated its superiority to differentiate between the two groups. Statistical analysis was conducted using MetaboAnalyst 3.0. Experimental data were presented as mean ± SD. One-way ANOVA was used for comparisons between groups, with statistical significance set at $P < 0.05$.

### *Evaluation of liver apoptosis using TUNEL staining*

The mice were categorized into four groups, comprising HBV-replicating mice and non-HBV mice. The HBV-replicating mice were allocated into two groups ($n$ = 6) for intraperitoneal treatment, administered once daily for 4 wk. Group 1 received NS, while group 2 was treated with high-dose LWWL (2 g/kg/d). Simultaneously, the non-HBV mice were also distributed into two groups ($n$ = 6) for intraperitoneal treatment over a 4-wk period. Group 1 received NS) while group 2 was treated with high-dose LWWL (2 g/kg/d). The liver tissue was used to assess apoptosis, with TUNEL staining

executed through the utilization of the TUNEL FITC Apoptosis Detection Kit[14].

### Evaluation of apoptosis in liver cell lines by flow cytometry

Apoptosis assessment was conducted using six distinct cell lines, consisting of three HBV-replicating cell lines (HepG2.2.15, HepG2.A64 and HepG2.1403F) as well as three non-HBV-replicating cell lines (LX2, HepG2 and L02). The six cell lines were individually seeded into six-well plates at $2 \times 10^5$ per well for Flow cytometry. Following 24 h incubation, each plate underwent treatment with 0.8 mg/mL LWWL for 72 h at 37 °C in a 5% $CO_2$ incubator. Subsequent to treatment, the cells were harvested, rinsed with ice-cold phosphate-buffered saline, and subjected to 5 min incubation with Annexin V-PE and 7-AAD (Multi Sciences, Hangzhou, China). Analysis was conducted utilizing a flow cytometer equipped with the FAC Station data management system and Cell Quest software (Becton Dickinson, San Jose, CA, United States). Data were analyzed using FlowJo v10.

### Statistical analysis

All statistical analyses were conducted using SPSS 16.0. The data are presented as mean ± SD and underwent analysis using a *t*-test for comparisons between groups. In instances where suitable, one-way ANOVA was used. Statistical significance was defined as $P < 0.05$.

## RESULTS

### Antiviral effect of LWWL in wild-type and drug-resistant HBV cell models

The cytotoxicity assay demonstrated that 0.8 mg/mL LWWL did not have any noticeable impact on cell viability. For HepG2.1403F cells, $CC_{50}$ was 6.13 mg/mL (Figure 1A). Evaluation of the antiviral effect of LWWL was conducted on days 1, 3 and 5, under safe concentrations. The optimal antiviral effect of LWWL was observed on day 5 in HepG2.1403F cells (Figure 1B).

In HepG2.1403F cells, 0.8 mg/mL LWWL exhibited inhibitory rates of 51.36%, 24.74% on HBV DNA and HBsAg respectively (Figure 1C). In HepG2.1403F cells, 200 μmol/L TDF showed inhibitory rates of 80.42%, 10.22% on HBV DNA and HBsAg respectively (Figure 1D). In HepG2.1403F cells, 0.8 mg/mL LWWL and 200 μmol/L TDF exhibited inhibitory rates of 50.74%, 40.02% on pgRNA respectively (Figure 1E). In HepG2.1403F cells, neither LWWL nor TDF demonstrated any inhibitory effect on cccDNA (Figure 1F). In HepG2.1403F cells, 0.8 mg/mL LWWL and 200 μmol/L TDF exhibited rates of 47.78%, 62.09% for pgRNA/cccDNA respectively (Figure 1G). In HepG2.215 cells, neither LWWL nor TDF demonstrated any inhibitory effect on cccDNA (Figure 1H). In HepG2.215 cells, 0.8 mg/mL LWWL and 200 μmol/L TDF exhibited rates of 39.51%, 54.15% for pgRNA/cccDNA respectively (Figure 1I). In HepG2.A64 cells, neither LWWL nor TDF demonstrated any inhibitory effect on cccDNA (Figure 1J). In HepG2.A64 cells, 0.8 mg/mL LWWL and 200 μmol/L TDF exhibited rates of 46.74%, 69.73% for pgRNA/cccDNA respectively (Figure 1K).

### Metabolomics analysis elucidated the apoptosis-related pathway through which LWWL acts against HBV

The results from the Base Peak Chromatogram (Figure 2A) and Boxplot (Figure 2B) indicated that the expression of total metabolites was consistent across all groups, meeting the requirements for intergroup metabolite comparison. As per the PCA results, the metabolic profiles of the control group (CG), low-dose LWWL group (LG), high-dose LWWL group (HG), and TDF group (TG) displayed distinct differences, highlighting effective differentiation among the groups. Of particular note was the distinct metabolic profile of HG/CG (Figure 2C). For a more refined identification of differentially expressed metabolites (DEMs), the metabolic profile between the two groups was refined through OPLS-DA, building upon the foundation provided by PCA to present a comprehensive metabolic overview of the four groups. OPLS-DA clearly demonstrated the distinct separation of the two groups (Figure 2D). The analyses of metabolic pathway enrichment and screening of DEMs were robustly corroborated by OPLS-DA and PCA.

The criteria used for identifying DEMs in this study included VIP > 1 and $P < 0.05$. In HG/CG, HG/LG, HG/TG, LG/CG, LG/TG and TG/CG groups, 107, 118, 162, 55, 26 and 94 distinct metabolites were identified, respectively (Figure 3A). Hierarchical clustering illustrated a significant distinction in DEMs between HG and CG, effectively discerning the two groups (Figure 3B). As indicated by KEGG analysis, the DEMs within HG/CG exhibited prominent enrichment in pathways such as taurine and hypotaurine metabolism, arginine biosynthesis, and the FoxO signaling pathway, alongside other pathways (Figure 3C).

This study focused on the DEMs within the HG/CG comparison. Through consultation of pertinent literature and functional annotations of metabolites, eight key DEMs-adenosine, D-galactose, L-lysine, L-isoleucine, L-glutamate, L-ornithine, taurocholic acid, and 7α-dihydroxy-5-cholestenoate were identified. Their respective AUC values were 1, 0.944, 0.889, 0.944, 1, 0.861, 0.833 and 1, respectively (Figure 3D). The eight key DEMs could potentially serve as pivotal metabolites contributing to the anti-HBV effects of LWWL. This supposition is supported by heat map analysis, which highlighted their notable specificity and sensitivity in distinguishing between HG and CG (Figure 3E). Among the eight major DEMs, taurocholic acid, L-ornithine and L-lysine exhibited a substantial correlation with apoptosis (Figure 3F).

### Transcriptomic analysis revealed the LWWL-induced apoptosis-related pathway against HBV

Transcriptomic analysis has been extensively described in our prior work, with a particular emphasis on elucidating apoptosis-related gene expression[8]. The transcriptomic results revealed 24 differentially expressed genes (DEGs) associated with anti-HBV effects. Through experimental validation, we identified 13 DEGs that were specifically related

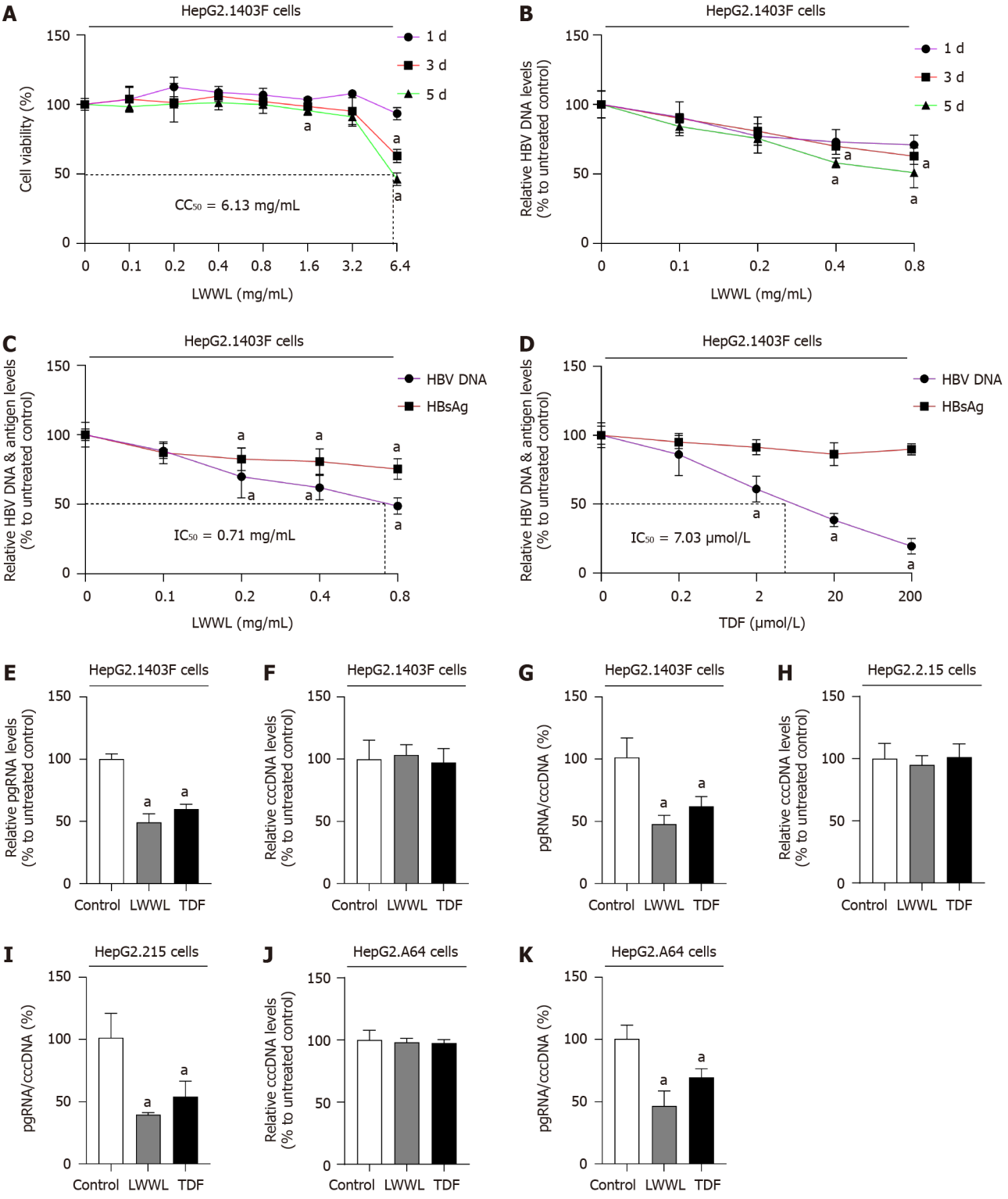
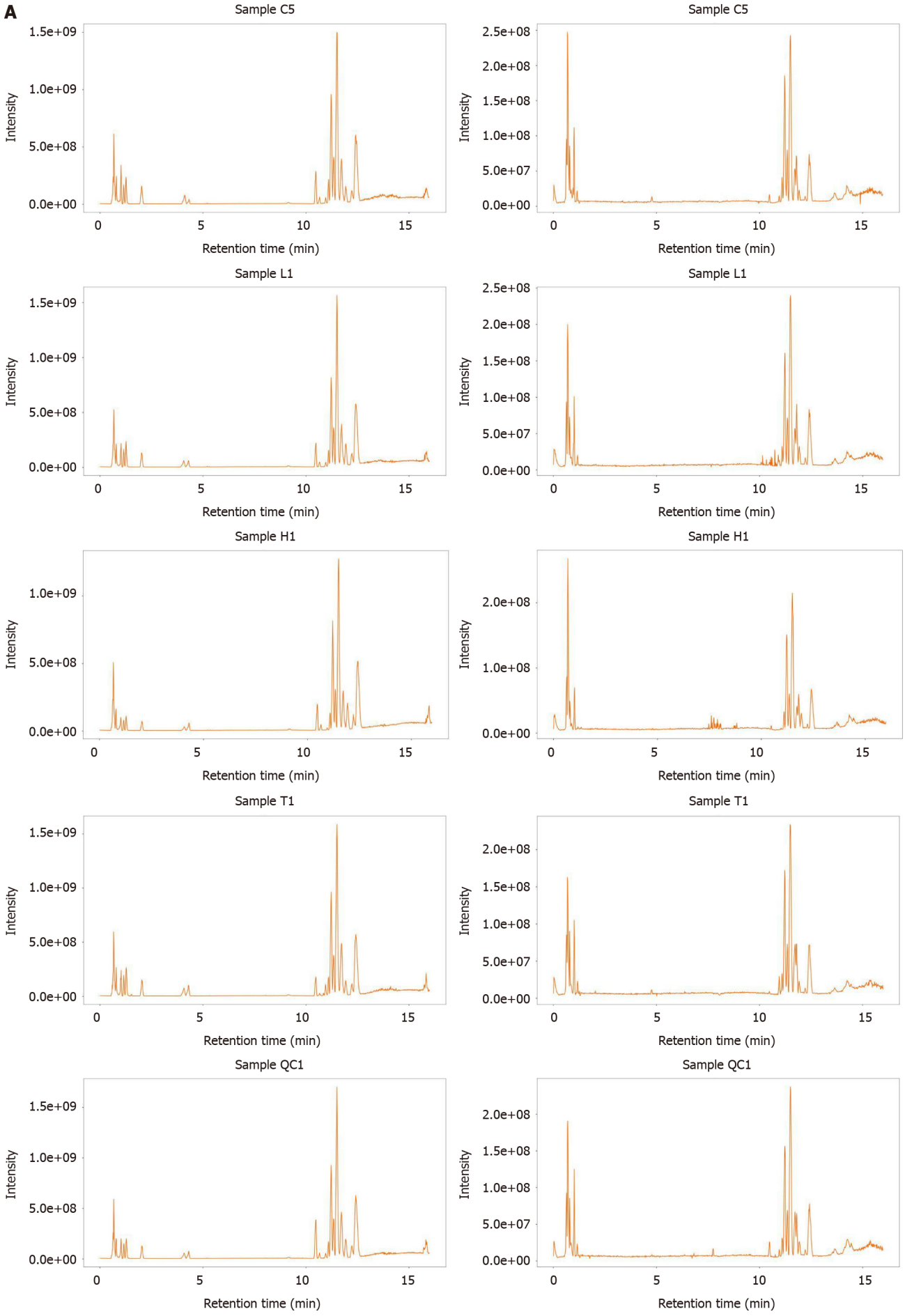



**Figure 1 Effects of Liuweiwuling Tablet on hepatitis B virus DNA/covalently closed circular DNA/RNA and antigen in cell models.** A: The safe concentration of Liuweiwuling Tablet (LWWL), defined as maintaining ≥ 95% cell viability compared with the untreated controls, was evaluated in HepG2.1403F cells; B: The optimal effective time for anti-hepatitis B virus (HBV) activity of LWWL, determined as the time-point demonstrating the strongest suppression of HBV DNA during a 5-d observation period, was evaluated in HepG2.1403F cells; C: The inhibitory effects of LWWL on HBV DNA and supernatant hepatitis B surface antigen (HBsAg) were evaluated in HepG2.1403F cells; D: The inhibitory effects of tenofovir disoproxil fumarate (TDF) on HBV DNA and supernatant HBsAg were assessed in HepG2.1403F cells; E: The inhibitory effects of both LWWL and TDF on pregenomic RNA (pgRNA) were measured in HepG2.1403F cells; F: The inhibitory effects of both LWWL and TDF on covalently closed circular DNA (cccDNA) were measured in HepG2.1403F cells; G: The inhibitory effects of both LWWL and TDF on pgRNA/cccDNA were measured in HepG2.1403F cells; H: The inhibitory effects of LWWL and TDF on cccDNA were evaluated in HepG2.2.15 cells; I: The inhibitory effects of LWWL and TDF on pgRNA/cccDNA were evaluated in HepG2.2.15 cells; J: The inhibitory effects of LWWL and TDF on cccDNA were investigated in HepG2.A64 cells; K: The inhibitory effects of LWWL and TDF on pgRNA/cccDNA were investigated in HepG2.A64 cells. $^{a}P < 0.05$ *vs* control.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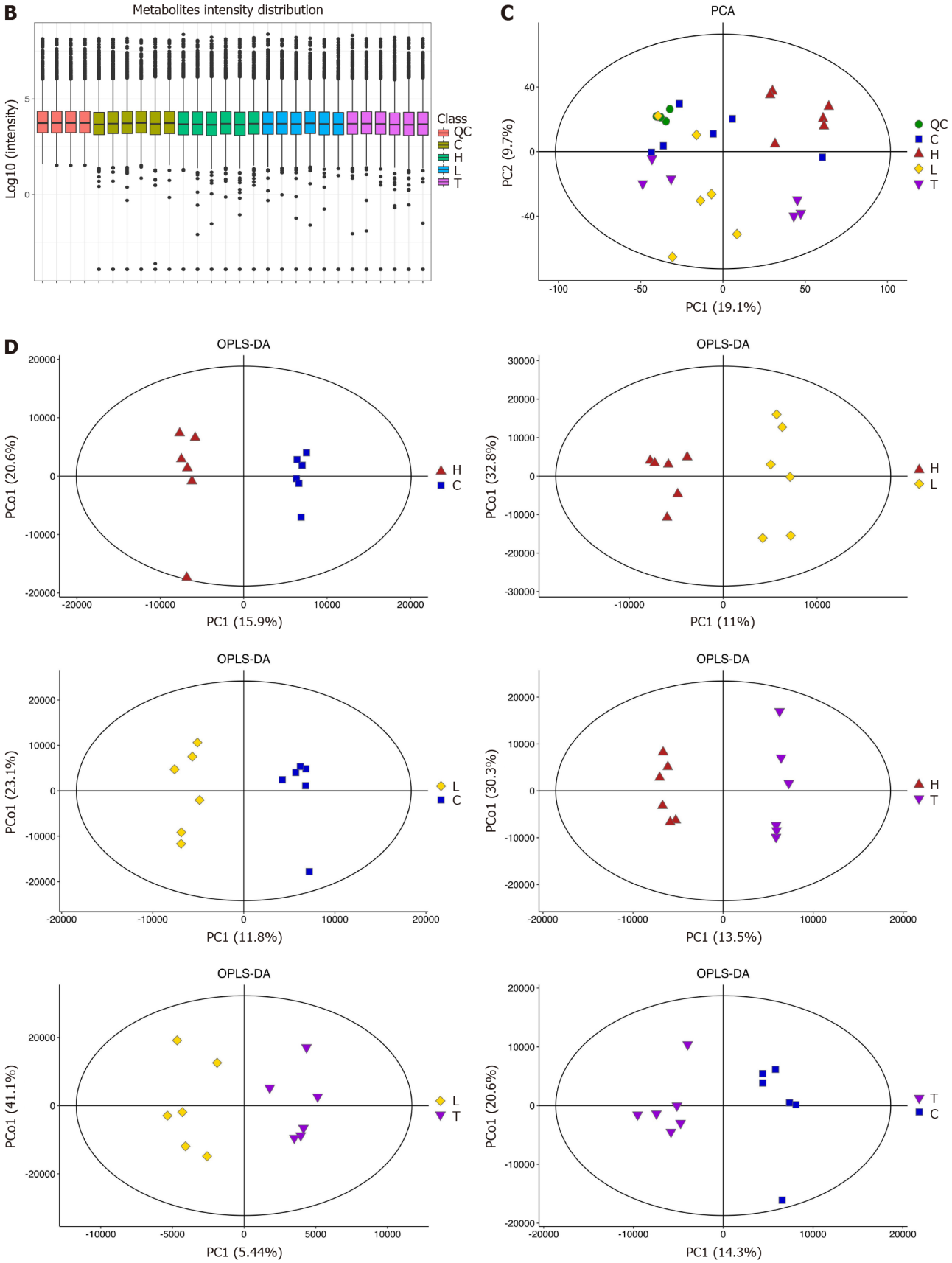
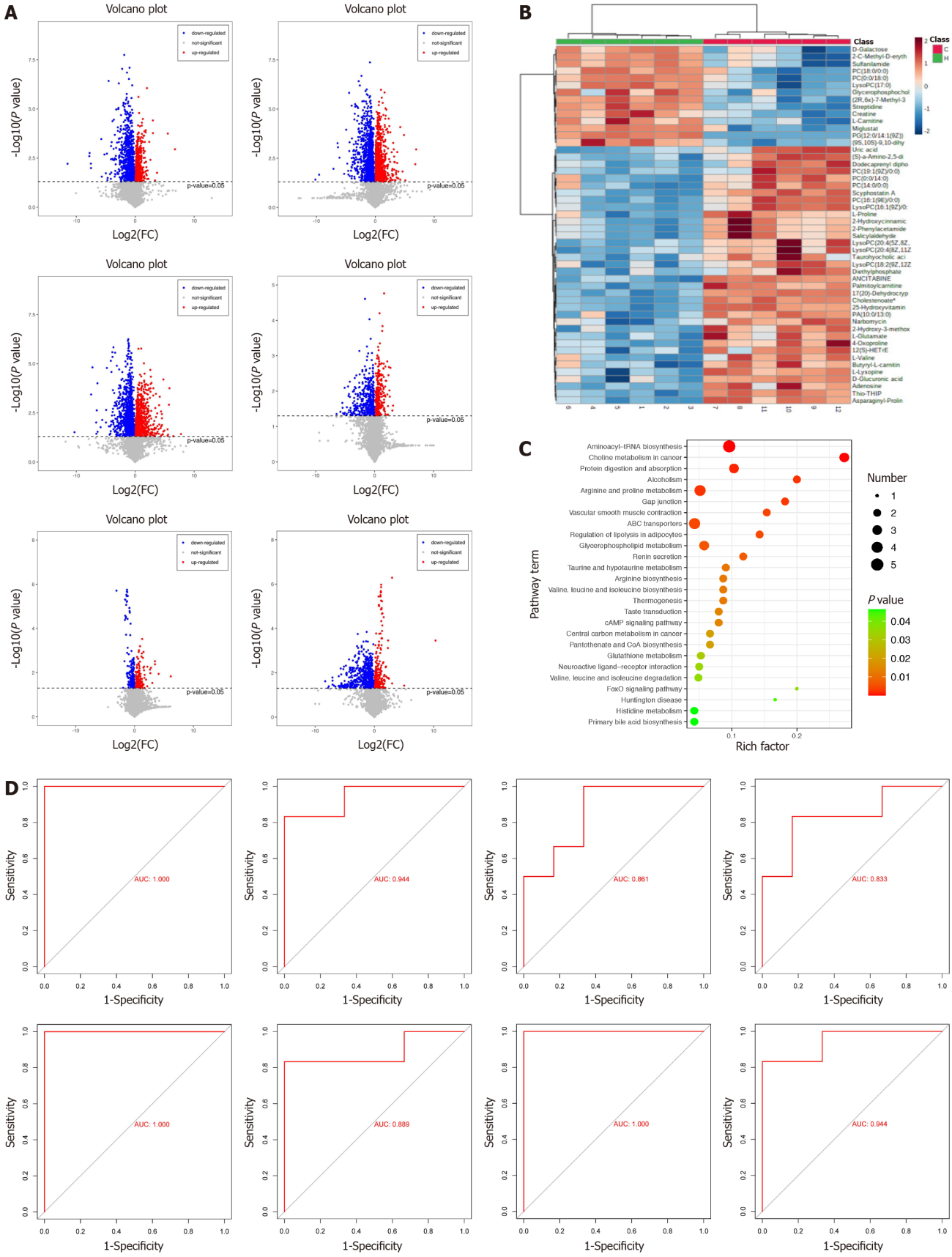



**Figure 2 Quality control analysis of serum metabolomics in pAAV-HBV1.2 replication mice treated with Liuweiwuling Tablet.** A: Ion base peak diagram; B: Distribution of metabolic sensitivity; C: Principal component analysis plot; D: Orthogonal partial least squares discriminant analysis plot. LWWL: Liuweiwuling Tablet; HG: High-dose LWWL group; LG: Low-dose LWWL group; CG: Control group; TG: Tenofovir disoproxil fumarate group. OPLS-DA: Orthogonal partial least squares discriminant analysis; PCA: Principal component analysis.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down-regulated
not-significant
up-regulated
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Aminoacyl−tRNA biosynthesis
Choline metabolism in cancer
Protein digestion and absorption
Alcoholism
Arginine and proline metabolism
Gap junction
Vascular smooth muscle contraction
ABC transporters
Regulation of lipolysis in adipocytes
Glycerophospholipid metabolism
Renin secretion
Taurine and hypotaurine metabolism
Arginine biosynthesis
Valine, leucine and isoleucine biosynthesis
Thermogenesis
Taste transduction
cAMP signaling pathway
Central carbon metabolism in cancer
Pantothenate and CoA biosynthesis
Glutathione metabolism
Neuroactive ligand−receptor interaction
Valine, leucine and isoleucine degradation
FoxO signaling pathway
Huntington disease
Histidine metabolism
Primary bile acid biosynthesis
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Sensitivity
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AUC: 0.861
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AUC: 1.000
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AUC: 1.000
AUC: 0.944

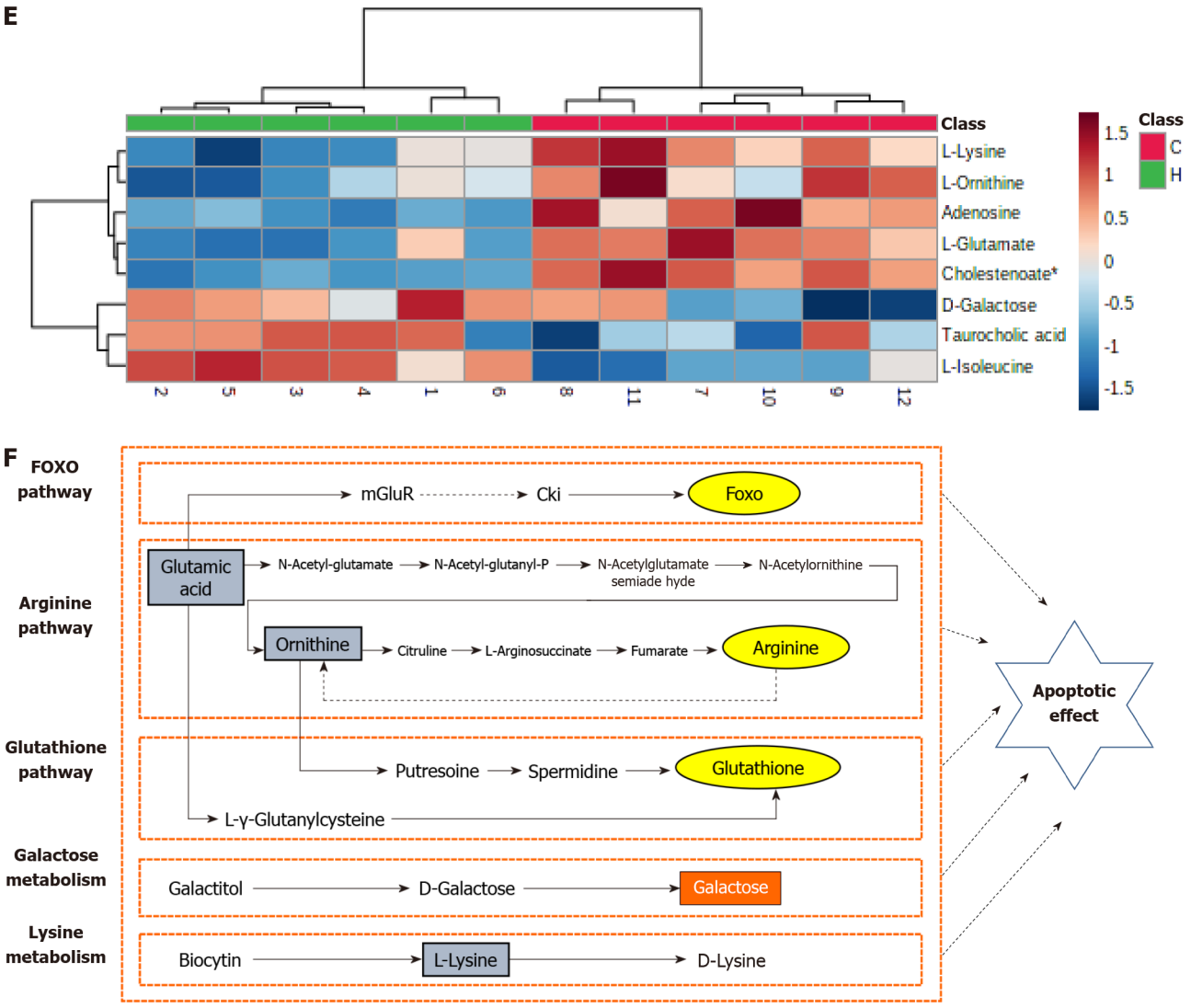


**Figure 3 Differential expression metabolites in serum metabolomics of pAAV-HBV1.2 replication mice treated with Liuweiwuling Tablet.** A: Volcanogram of differentially expressed metabolites (DEMs); B: Heat map of DEMs between High-dose Liuweiwuling Tablet group (HG)/control group (CG); C: Kyoto Encyclopedia of Genes and Genomes pathway enrichment for the DEMs between HG/CG; D: Receiver operating characteristic curve validation of the DEMs between HG/CG; E: Heat map of eight key DEMs between HG/CG; F: Potential apoptosis-related metabolic pathways of Liuweiwuling Tablet against hepatitis B virus. LWWL: Liuweiwuling Tablet; HG: High-dose LWWL group; LG: Low-dose LWWL group; CG: Control group; TG: Tenofovir disoproxil fumarate group.

to anti-HBV activity. Thirteen DEGs between the LWWL group and the CG were confirmed using reverse transcriptase-PCR. In comparison with the CG, the LWWL group exhibited significantly lower expression levels of EGR2 and FOS, while the expression of IKBKE, CCNE2, AKT3, CREB3L2, PIK3R3, CREBBP, BCL2, PCNA, E2F1, CASP8 and P53 was significantly higher. Among these 13 genes, *AKT3*, *PIK3R3*, *BCL2*, *PCNA*, *FOS*, *CASP8* and *P53* were associated with the PI3K-AKT, CASP8-CASP3 and P53 pathways; all of which are apoptosis related pathways (Table 1).

### *Apoptotic effect of LWWL on HBV-replicating and non-HBV-replicating cell models*

Based on analysis of apoptosis flow, the apoptosis rate in the LWWL-treated group was significantly increased compared with the CG in HepG2.2.15 cells (2.92% ± 1.01% *vs* 6.68 ± 2.04%, $P < 0.05$), HepG2.A64 cells (4.89% ± 1.28% *vs* 8.52% ± 0.50%, $P < 0.05$) and HepG2.1403F cells (3.76% ± 1.40% *vs* 7.57% ± 1.35%, $P < 0.05$) (CG *vs* LWWL-treated group) (Figure 4). However, no notable difference in apoptosis rate was observed between the LWWL-treated and CG in HepG2 cells (5.04% ± 0.74% *vs* 5.51% ± 1.57%, $P > 0.05$), L02 cells (5.49% ± 0.80% *vs* 5.48% ± 1.01%, $P > 0.05$) and LX2 cells (6.29% ± 1.54% *vs* 6.29% ± 0.88%, $P > 0.05$) (CG *vs* LWWL-treated group) (Figure 5).

### *Apoptotic effect of LWWL on HBV-replicating and non-HBV-replicating mouse models*

TUNEL staining was used to assess the impact of LWWL on liver apoptosis in the HBV and non-HBV-replicating mouse models. In the HBV-replicating mouse model, LWWL enhanced the apoptotic response of the liver as compared with the CG. In the non-HBV-replicating mouse model, LWWL had no significant apoptotic effect on the liver when compared with the CG (Figure 6).

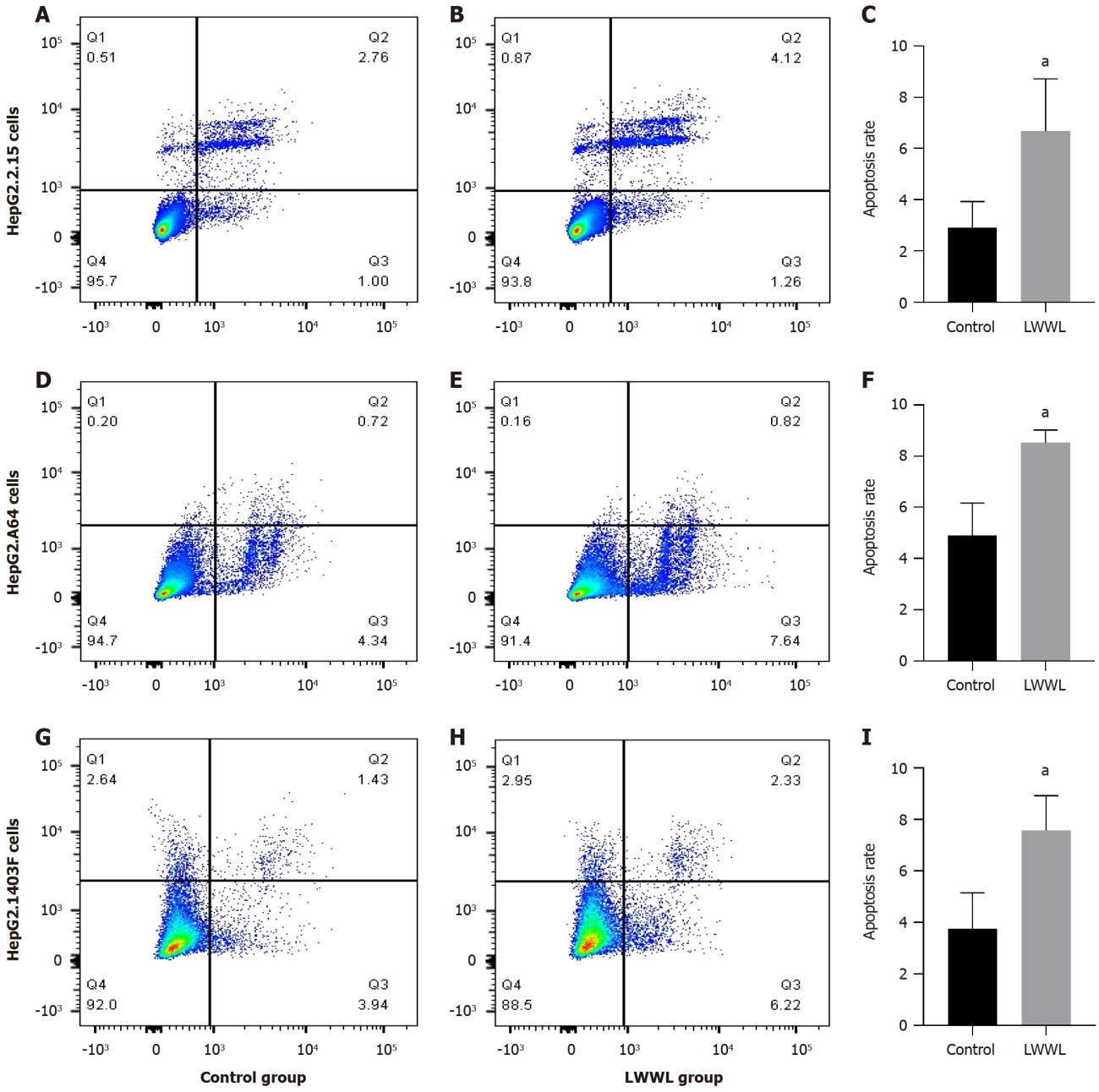


**Figure 4 Apoptotic effect of Liuweiwuling Tablet in hepatitis B virus cell models.** A: Apoptotic effect of Liuweiwuling Tablet (LWWL) in HepG2.2.15 cells; B: Apoptotic evaluation of control group in HepG2.2.15 cells; C: Statistical analysis of apoptosis in HepG2.2.15 cells; D: Apoptotic effect of LWWL in HepG2.A64 cells; E: Apoptotic evaluation of control group in HepG2.A64 cells; F: Statistical analysis of apoptosis in HepG2.A64 cells; G: Apoptotic effect of LWWL in HepG2.1403F cells; H: Apoptotic evaluation of control group in HepG2.1403F cells; I: Statistical analysis of apoptosis in HepG2.1403F cells. $^{a}P < 0.05$ *vs* control. LWWL: Liuweiwuling Tablet.

## DISCUSSION

Previously, we assessed the antiviral efficacy of LWWL in animal and cell models of wild-type HBV infection, using established virological indicators such as HBV DNA, HBsAg, HBeAg, and hepatitis B core antigen[8]. To provide a more comprehensive assessment of the anti-HBV potential of LWWL, the present study expanded to encompass a multidrug-resistant HBV cell model in addition to the wild-type HBV cell model. The evaluation of novel HBV indicators including pgRNA, cccDNA and pgRNA/cccDNA were integrated alongside conventional virological markers. LWWL demonstrated robust inhibitory efficacy against both wild-type and drug-resistant HBV. When compared with the TDF control, LWWL was more effective at suppressing HBV antigens and transcriptional activity (pgRNA/cccDNA) rather than HBV DNA level.

We extended our comprehensive assessment of the activity of LWWL against HBV and used a combined approach of transcriptomics and metabolomics to delve into its underlying mechanism, revealing a predominant involvement of apoptosis-related pathways. Transcriptomics and metabolomics revealed that the anti-HBV effect of LWWL primarily involved apoptosis-associated pathways, encompassing the PI3K-AKT, CASP8-CASP3 and P53 pathways, in conjunction

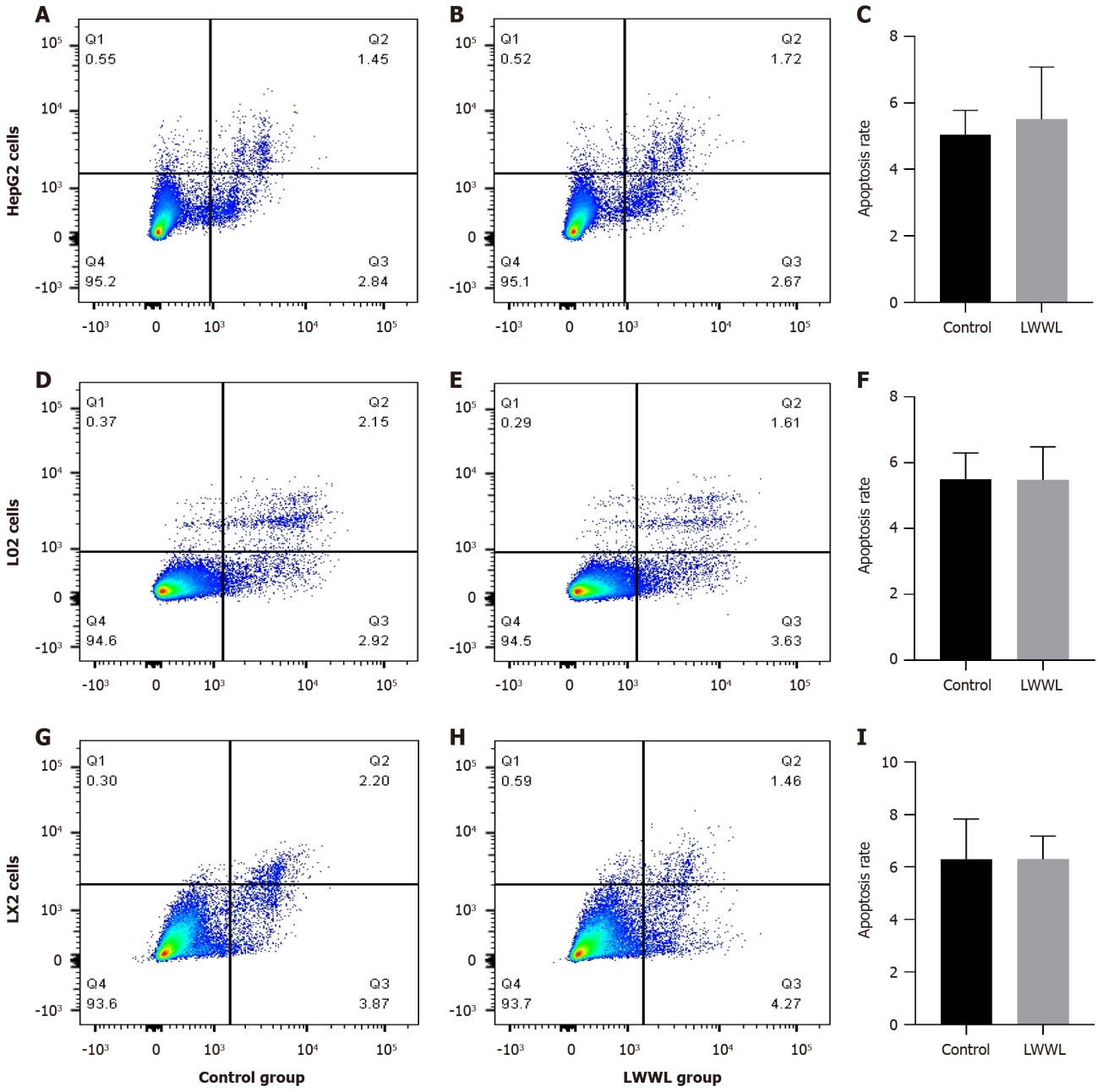


**Figure 5 Apoptotic effect of Liuweiwuling Tablet in non- hepatitis B virus cell models.** A: Apoptotic effect of Liuweiwuling Tablet (LWWL) in HepG2 cells; B: Apoptosis in the control group is presented in HepG2 cells; C: Statistical analysis of apoptosis is provided in HepG2 cells; D: Apoptotic effect of Liuweiwuling Tablet in L02 cells; E: Apoptosis in the control group is presented in L02 cells; F: Statistical analysis of apoptosis is provided in L02 cells; G: Apoptotic effect of LWWL in LX2 cells; H: Apoptosis in the control group is presented in LX2 cells; I: Statistical analysis of apoptosis is provided in LX2 cells. LWWL: Liuweiwuling Tablet.

with apoptosis-related metabolites including D-galactose, lysine, L-glutamic acid and ornithine[8]. Subsequent examination of the terminal outcomes of the apoptosis-related pathways of LWWL revealed that LWWL selectively triggered apoptosis in HBV-infected cells but had no effect on non-HBV-infected hepatic cells *in vitro* and *in vivo*.

The pathogenesis of CHB is complex. Alongside the extensively studied immunological mechanism, the apoptotic pathway of HBV has progressively gained prominence as a focal area of interest. Following HBV infection of liver cells, the equilibrium of the body is disrupted, culminating in diverse disorders, among which, disruption of apoptosis homeostasis plays a pivotal role. HBV inhibits liver cell apoptosis to sustain its conducive survival microenvironment[4,15,16]. By building upon this foundation, some investigations have delved into the anti-HBV mechanism of drugs through the modulation of apoptosis-associated pathways. Notably, polysaccharides derived from *Sipunculus nudus* and humic acid were found to modulate the CASP3 pathway[17,18], while Folium Artemisiae Argyi exhibited the ability to regulate the Wnt/β-catenin pathway[19], inducing apoptosis in a dose-dependent manner in HepG2.2.15 cells and hindering HBV DNA replication. Ascentage Pharma APG-1387, currently advancing to phase II clinical trials, stands as a cutting-edge anti-HBV therapeutic that centers on stimulating the apoptosis of HBV-infected cells[20]. Consequently, the exploration and development of anti-HBV drugs through the lens of apoptosis is scientifically important and clinically relevant. In contrast to prior medications that induce anti-HBV effects *via* apoptosis induction, we have unearthed an

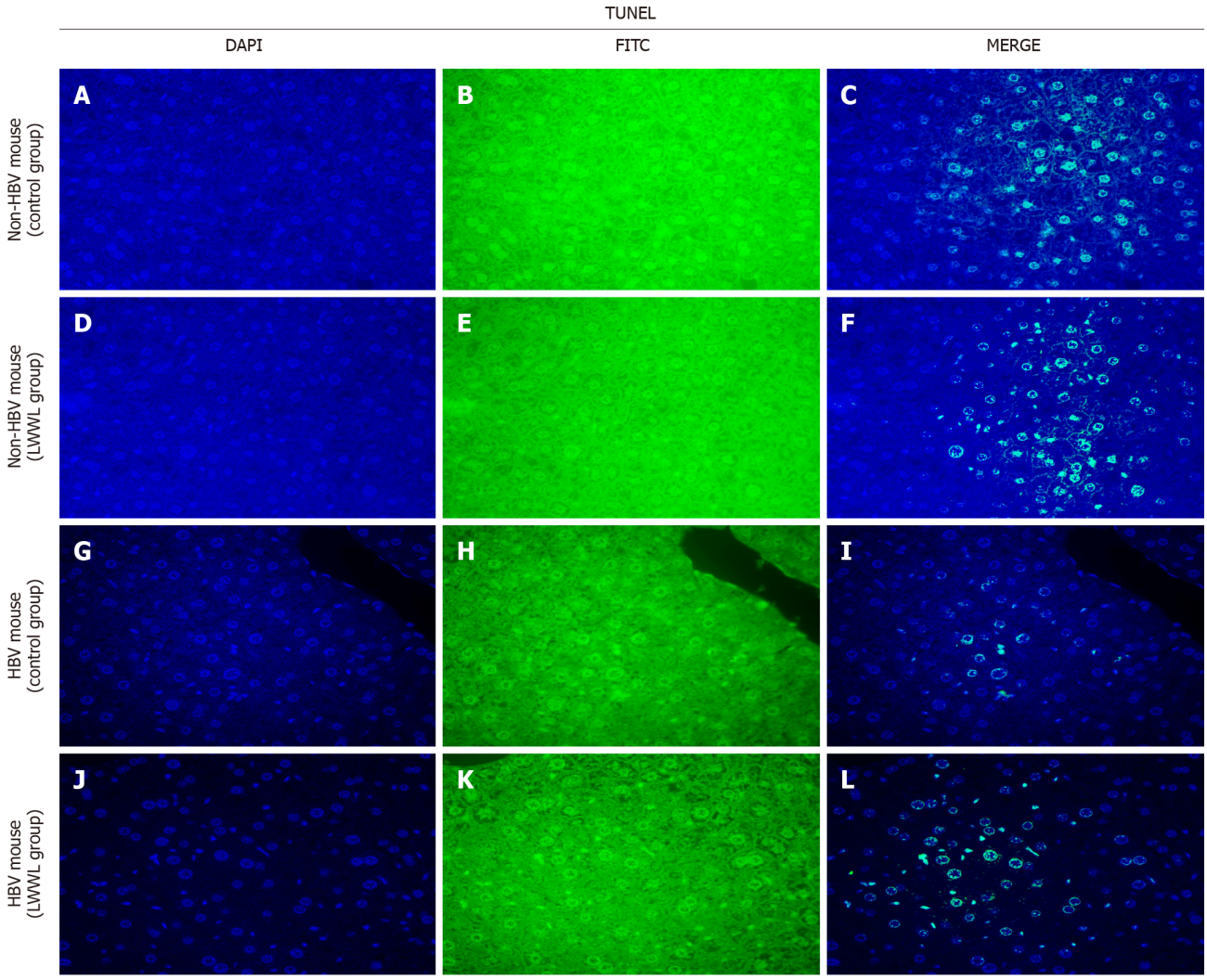


**Figure 6 Apoptotic effect of Liuweiwuling Tablet in the liver of hepatitis B virus (HBV) and non-HBV-replicating mouse models.** A-C: Liver apoptosis in the control group for the non- hepatitis B virus (HBV)-replicating mouse model; D-F: Liver apoptosis in the Liuweiwuling Tablet (LWWL)-treated group in the non-HBV-replicating mouse model; G-I: Liver apoptosis in the CG for the HBV-replicating mouse model; J-L: Liver apoptosis in the LWWL-treated group in the HBV-replicating mouse model. LWWL: Liuweiwuling Tablet; HBV: Hepatitis B virus.

innovative selective apoptosis mechanism against HBV. This signifies that LWWL can target and eliminate HBV-infected cells while sparing normal cells. This may account for the favorable safety profile of LWWL in clinical settings. The activation of apoptosis-associated pathways (PI3K-AKT, CASP8-CASP3 and P53) by LWWL likely intertwines with the modulation of apoptosis-related metabolites (D-galactose, lysine, L-glutamate and ornithine), resulting in apoptosis of HBV-infected hepatic cells (Figure 7).

## CONCLUSION

In conclusion, our study establishes, for the first time, the potent HBV-suppressive capability of the Chinese patent medicine LWWL. These suppressive effects surpassed those of TDF in terms of antigen expression *in vitro* and *in vivo*. A foundational insight into the mechanism of the anti-HBV action of LWWL suggests that it selectively induces apoptosis in HBV-replicating hepatic cells while not affecting non-HBV-replicating hepatic cells. These findings have revealed novel insights into the anti-HBV potential of LWWL, offering a prospect of enhancing combination therapy with LWWL and established NAs. Additionally, these insights could serve as a novel mechanistic reference for the advancement of anti-HBV medications.

**Table 1 Differentially expressed genes in Liuweiwuling Tablet anti-hepatitis B virus effects identified through transcriptomic analysis and validated by real-time reverse transcriptase-PCR**

| Gene | Fold change | *P* value | Is it related with apoptosis |
|---|---|---|---|
| *E2F1* | 4.44770094 | 0.008685393 | No |
| *PCNA* | 4.047933076 | 0.002898892 | No |
| *CASP8* | 3.570355797 | 0.003000082 | Yes |
| *TP53* | 3.452487306 | 0.016679865 | Yes |
| *CREBBP* | 2.885252648 | 0.003455666 | No |
| *IKBKE* | 2.849255028 | 0.016173753 | No |
| *PIK3R3* | 2.815615387 | 0.011672063 | Yes |
| *CCNE2* | 2.506258474 | 0.014722413 | No |
| *BCL2* | 2.239207884 | 0.007566661 | Yes |
| *AKT3* | 2.023770673 | 0.003487678 | Yes |
| *CREB3L2* | 0.621889559 | 0.006920633 | No |
| *EGR2* | 0.44857779 | 0.001957335 | No |
| *FOS* | 0.183742709 | 0.02708347 | Yes |

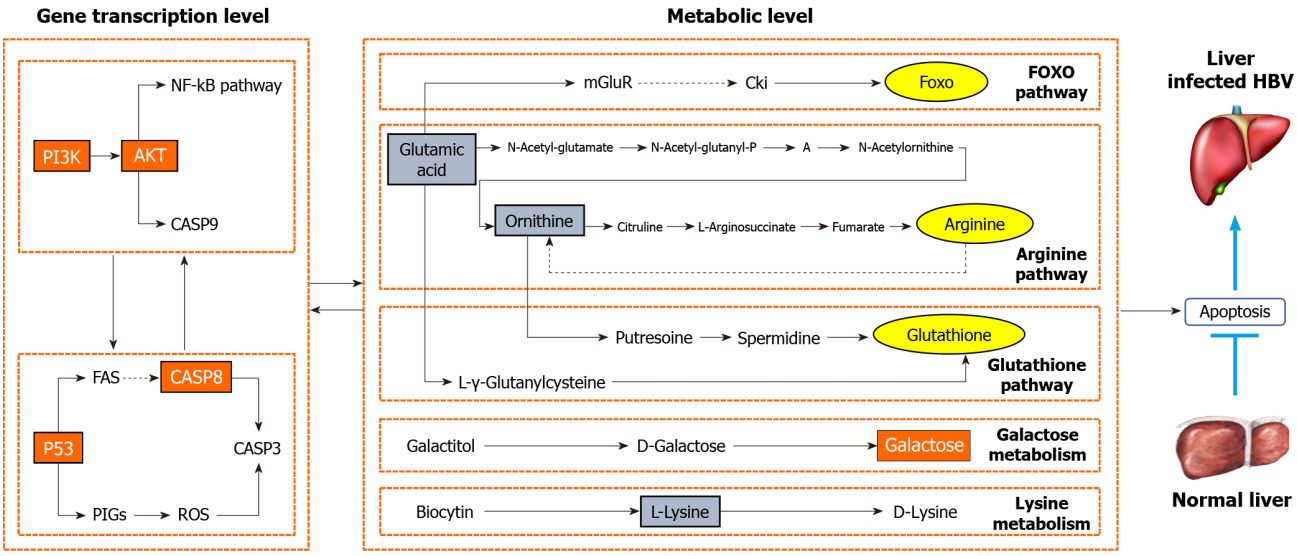


**Figure 7 Possible selective apoptosis pathways of Liuweiwuling Tablet against hepatitis B virus.** HBV: Hepatitis B virus.

## ARTICLE HIGHLIGHTS

### *Research background*

Nucleoside/nucleotide analogs (NAs) are the most commonly-used anti-hepatitis B virus (HBV) agents. They effectively inhibit viral replication, whereas the suppressive effects are much weaker on HBV antigen and drug-resistant HBV. Therefore, it is very important and meaningful to find new drugs to make up for the deficiency of NAs. Liuweiwuling Tablet (LWWL) is a licensed Chinese patent medicine for anti-inflammation of chronic HBV infection. We previous found that LWWL has an anti-HBV effect in wild-type HBV model for the first time, but the mechanism is still unclear.

### *Research motivation*

The objective of this study was to elucidate the scientific significance of LWWL's antiviral advantages, in order to provide a reference for the clinical application of LWWL against HBV.

### *Research objectives*

The study aimed to explore the potential mechanism of anti-HBV, and further comprehensively evaluate its anti-HBV effect in drug-resistant HBV model.

### Research methods
*In vitro* experiments utilized three HBV-replicating cell lines and three non-HBV-replicating cell lines, while an *in vivo* experiment involved a hydrodynamic injection-mediated mouse model with HBV replication. Transcriptomics and metabolomics were employed to investigate the underlying mechanisms.

### Research results
Our study establishes the potent HBV-suppressive capability of the Chinese patent medicine LWWL. Furthermore, these suppressive effects surpassed those of tenofovir disoproxil fumarate in terms of antigen expression in both *in vitro* and *in vivo*. A foundational insight into the mechanism of LWWL's anti-HBV action suggests its involvement in the regulation of selective apoptosis, selectively inducing apoptosis in HBV-replicating hepatic cells while not affecting non-HBV-replicating hepatic cells.

### Research conclusions
The preliminary revelation in the anti-HBV pharmacological mechanism is that LWWL exerts a potent inhibitory impact on both wild-type and drug-resistant HBV, potentially involving a selective regulation of apoptosis. These findings offer novel insights into the anti-HBV activities of LWWL and present a novel mechanism for the development of anti-HBV medications.

### Research perspectives
Novel anti-HBV drugs were developed by using a selective regulation of apoptosis mechanism.

## FOOTNOTES

**Co-first authors:** Fei-Lin Ge and Yan Yang.

**Co-corresponding authors:** Yan Liu and Xiao-He Xiao.

**Author contributions:** Liu Y and Xiao XH made substantial contributions to the conception or design of the study; Ge FL and Yang Y were in charge of the acquisition, analysis and interpretation of data, and wrote the manuscript; Li YH, Cao MZ and Wang J participated in experiments related to cell and animal models; Bai ZF, Ren ZG and Si LL were responsible for revision of the manuscript. All authors finally read and approved the version to be published. Ge FL and Yang Y contributed equally to this work as co-first authors. The reasons are the following. First, the research was performed as a collaborative effort, and the designation of co-first authors authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. Second, co-first authors contributed efforts of equal substance throughout the research process. Liu Y and Xiao XH contributed equally to this work as co-corresponding authors. The reasons are the following. First, they played a key role in coordinating the research team. Second, they made a great contribution to the original innovation of the article. In summary, we believe that designating Ge FL and Yang Y as co-first authors, Liu Y and Xiao XH as co-corresponding authors is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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**Country/Territory of origin:** China

**ORCID number:** Fei-Lin Ge 0000-0002-0992-474X; Lan-Lan Si 0000-0003-0222-5169; Jun Wang 0000-0002-6261-8995; Zhao-Fang Bai 0000-0002-6208-150X; Zhi-Gang Ren 0000-0003-0798-3444; Xiao-He Xiao 0000-0002-3719-6575; Yan Liu 0000-0003-1498-4314.

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LETTER TO THE EDITOR

# Hepatic perivascular epithelioid cell tumors: The importance of preoperative diagnosis

Shuai Yan, Jia-Jie Lu, Lin Chen, Wei-Hua Cai, Jin-Zhu Wu

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**Shuai Yan, Jia-Jie Lu, Wei-Hua Cai, Jin-Zhu Wu,** Department of Medical School, Nantong University, Nantong 226300, Jiangsu Province, China

**Shuai Yan, Jia-Jie Lu, Wei-Hua Cai, Jin-Zhu Wu,** Department of Hepatobiliary Surgery, Affiliated Nantong Hospital 3 of Nantong University, Nantong 226006, Jiangsu Province, China

**Lin Chen,** Nantong Institute of Liver Disease, Affiliated Nantong Hospital 3 of Nantong University, Nantong 226006, Jiangsu Province, China

**Corresponding author:** Jin-Zhu Wu, MD, Chief, Chief Doctor, Professor, Department of Hepatobiliary Surgery, Affiliated Nantong Hospital 3 of Nantong University, Nantong University, No. 19 Qixiu Road, Chongchuan District, Nantong 226300, Jiangsu Province, China. wjz1258@163.com

## Abstract

Accurate preoperative diagnosis is highly important for the treatment of perivascular epithelioid cell tumors (PEComas) because PEComas are mainly benign tumors and may not require surgical intervention. By analyzing the causes, properties and clinical manifestations of PEComas, we summarize the challenges and solutions in the diagnosis of PEComas.

**Key Words:** Hepatic perivascular epithelioid cell tumors; Liver; Preoperative diagnosis; Angiomyolipomas; Mesenchymal tissue-derived tumors



**Core Tip:** Hepatic perivascular epithelioid cell tumors (PEComas) are mesenchymal tumors composed of histologically and immunohistochemically unique perivascular epithelioid cells. They have nonspecific clinical manifestations, inconspicuous and variable imaging features and complex pathological phenotypes, which make preoperative diagnosis very difficult. By enumerating the practical problems faced by clinicians in the diagnosis and treatment of PEComa patients, we analyzed the methods and ideas used to improve the accuracy of preoperative diagnosis of PEComas and provided new insights into the choice of conservative treatment and surgical treatment.

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## TO THE EDITOR

We read the recently published papers of Kou *et al*[1] and express our satisfaction and congratulations on their excellent work. This is a well-written case report. By introducing three rare cases of hepatic perivascular epithelioid cell tumors (PEComas), they further noted the practical challenges faced by clinicians in the face of hepatic PEComas (HPEComas). We fully understand Kou *et al*'s concerns about the preoperative misdiagnosis of HPEComas[1]. Although the accuracy of a single examination may be insufficient to meet diagnostic requirements, combined examination of multiple imaging and immunohistochemical markers may be an effective method for improving accuracy. Accurate preoperative diagnosis is highly important for the treatment of HPEComas because PEComas are mainly benign tumors and may not require surgical intervention. Blind surgery without adequate diagnosis may introduce unnecessary treatment risks to patients.

In 2002, the World Health Organization (WHO) formally defined "PEComas" as mesenchymal tumors composed of histologically and immunohistochemically unique perivascular epithelioid cells (PECs), and PEComas include many different clinicopathological entities. Among them are angiomyolipoma, lymphangioma, lymphangioleiomyomatosis, clear cell tumor sugar and tumor types not otherwise specified[2]. Although PEComas and angiomyolipomas (AMLs) are theoretically subordinate, many clinical experts believe that the two are actually different manifestations of the same disease[3]. Therefore, in the following, we will discuss AMLs and PEComas as unified concepts and no longer make a special distinction.

## DEFINITION AND TUMOR PROPERTIES

It is currently clear that PEComas are mesenchymal tissue-derived tumors that are usually composed of blood vessels, smooth muscle and adipocytes[4]. However, the proportions of these tissue components tissue components differ among patients[5]. These patients often complain of abdominal discomfort but do not present accompanying abnormal serological test results[6-8]. At present, the pathogenesis of this tumor has not been elucidated[5,6]. Although more than 50% of renal AMLs are associated with tuberous sclerosis (TSC), it is estimated that only 5%-15% of patients with solitary liver tumors have such a link[4]. The vast majority of PEComas are benign, and malignant forms are extremely rare[9]. In view of the relatively few reports on malignant PEComas, a clear malignant standard has not yet been established[9]. However, even if PEComas are identified as benign, their boundary with malignant tumors is not very clear. First, cytological atypia, a characteristic of malignant tumors, is present in benign liver PEComas[10], while the consistent characteristic of malignant liver PEComas is considered to be coagulative necrosis[9]. Therefore, only when the tumor has necrosis, a large mass (> 10 cm), CD117 negativity, invasive behavior or other clinical evidence may it be considered a malignant liver PEComa[10]. Second, it has been reported that benign PEComas may even undergo malignant transformation during development, eventually displaying sarcomatoid or cancer-like characteristics[11]. Moreover, some patients have also been found to have advanced metastasis many years after the diagnosis of primary benign tumors[11]. Furthermore, in a recently proposed classification system, Folpe *et al*[12] divided PEComas into benign, uncertain malignant potential (UMP) and malignant tumor categories. We think that this classification may be appropriate because it identifies a variety of tumor behaviors shown during the development of PEComas, clearly specifying UMP and recognizing that malignant tumors may occur given malignant behaviors or features. When there are multiple malignant changes, the tumor can be defined as malignant. Finally, regarding the factors that lead to the malignant transformation of PEComas, several hypotheses have been proposed. It has been reported that malignant behavior occurs mainly in epithelioid PEComas and can be observed in the early stage of tumorigenesis[13]. The diagnosis of epithelioid PEComa in a patient by clinical examination indicates that there is a greater possibility of malignant transformation, and a treatment strategy for malignant tumors should be provided.

## PREOPERATIVE DIAGNOSIS

As discussed by Kou *et al*[1], PEComas have a very high preoperative misdiagnosis rate. According to Yang *et al*[14], Zeng *et al*[15] and Jung *et al*[16], only 18%-26% of patients with histopathologically confirmed PEComas were correctly diagnosed before surgery. This phenomenon may be due to many factors.

### *Imaging examination*

In terms of preoperative imaging data, according to two case reports covering 92 patients[17] and 94 patients[18], the accuracy of ultrasound was 0%-33%, that of computed tomography (CT) was 15.7%-18.2%, and that of magnetic resonance imaging (MRI) was 4.3%-22.7%. This may be due to the variability of the proportion and distribution of different tissue components on the image, hindering the diagnosis[8]. For example, the most prominent imaging features

of PEComas are mature adipose tissue and central thick-walled blood vessels[4]. This makes PEComas that are characterized by adipose tissue easy to diagnose. However, PEComas are variable and can also manifest as tumors containing low-fat tissue or nonfat tissue[4]. Moreover, the presence of fats has been found to be unreliable because some hydrocarbons contain fat, and these fats may also mimic PEComas during presentation[7]. This will negatively affect imaging experts and easily lead to incorrect diagnoses. To solve this problem, many clinical experts have adopted various approaches. For example, Ding *et al*[19], through the combined examination of ultrasound, CT, MRI and angiography in 79 patients, achieved a diagnostic accuracy of 52%. Wang *et al*[20] used complementary B-ultrasound and contrast-enhanced ultrasound (CEUS) to distinguish PEComas from other benign liver tumors. This may suggest that the combined examination of multiple images can improve the diagnostic rate. In a recent report, positron emission tomography (PET)/CT appeared to be an effective tool for diagnosing PEComas. The authors reported that PEComas exhibited strong 68Ga-FAPI uptake and slight 18F-FDG activity. This means that 68Ga-FAPI PET/CT has the potential to become a diagnostic tool for PEComas[21].

### *Laboratory examination*

Preoperative laboratory tests may only meet the requirements for excluding certain diseases. For example, in the three patients reported by Kou *et al*[1], except for the increase in CA-125 in Patient 3 with an ovarian tumor, the patients did not have abnormal serum tumor marker levels, which was consistent with previous findings that PEComas were not accompanied by abnormal serological results[7,8]. This approach may help clinicians rule out the diagnosis of some common tumors or simply make them doubt the proposed diagnosis.

### *Liver biopsy*

However, in a recent multicenter study, even histological analysis of preoperative liver biopsy data yielded a misdiagnosis rate of approximately 15%[22]. However, liver biopsy is still the best way to determine the diagnosis of such liver lesions before surgery. The presence of adipose tissue is helpful for distinguishing this disease from other malignant entities. However, due to the variability of the lesion and the small amount of tissue obtained by puncture, the fat area may be sampled or not, making diagnosis from puncture biopsies challenging[3]. However, compared with that of conventional imaging, the diagnostic accuracy of biopsy has increased considerably. Notably, almost all the PEComas were strongly positive for Human melanoma Black-45 (HMB-45), S-adenosyl methionine (SAM) and melan-A[23-25]. Ameurtesse *et al*[26] also reported that HMB-45 cells were generally positive; melan-A and SMA were frequently expressed. The negative expression of S100, desmin and vimentin may be specific signs of HPEComas. If preoperative puncture or intraoperative frozen pathological examination can comprehensively account for the difference between the imaging and microscopic examination results of such patients and common tumors and if HMB-45, Melan-A and other rare liver cancer histopathological immunohistochemical indicators are used, the accuracy of preoperative diagnosis may also increase considerably.

## PREOPERATIVE DIFFERENTIAL DIAGNOSIS

According to the misdiagnosis results, the main preoperative misdiagnosis of liver PEComas is hepatocellular carcinoma (HCC)[27]. The reasons are diverse. The multiple components of PEComas vary among individuals, and the proportion of fat and hemangioma components in the tumor volume varies from less than 10% to more than 90%[28,29]. Variable imaging results can confound the diagnosis and thus increase the probability of misdiagnosis as common HCC[10]. As mentioned above, benign PEComas are characterized by cytological atypia and are easily confused with other malignant tumors. The most common confounding factor is HCC[30]. As a representative malignant PEComa, epithelioid angiomyolipoma (EAML) does not contain or contains only a small amount of eye fat; this feature manifests as arterial enhancement and delayed washout and is also consistent with the general characteristics of HCC. Even if there are many complex disturbance factors, the identification of HCC and PEComas is not straightforward. First, unlike in general, the patient's conventional serum tumor marker, hepatitis marker, and alpha-fetoprotein results are negative. Second, in imaging, compared with HCC, PEComas lack a capsule, have reduced peripheral enhancement of the tumor, and may not exhibit use of the portal vein as a feature of their drainage, which might otherwise facilitate identification[31-33]. In a recent study, gadoxetic acid-enhanced MRI was also used to distinguish PEComas from HCC[34]. Kim *et al*[34] reported that 100% of PEComas and 85% of HCCs showed arterial enhancement and delayed washout on gadoxetic acid-enhanced MRI. Compared with HCC, PEComas showed a greater frequency of homogeneous low signals in delayed hepatobiliary phase (HBP) imaging (83% *vs* 41%). These authors believe that this is due to the lack of hepatocytes in PEComas, which results in a more uniform low SI on HBP images, while HCC may contain some poorly developed hepatocytes, resulting in more uneven high signal intensity on HBP images. Therefore, HBP examination *via* GA-enhanced MRI will be a powerful way to differentiate PEComas from HCC. Finally, due to the rarity of PEComas, many pathologists or imaging experts are not familiar with these tumors, leading to the most common HCC often being considered the final result. However, although the clinical and radiological features of these lesions often overlap, careful observation of histological clues can help to eliminate various diseases of the same species to obtain the most accurate diagnosis.

## TREATMENT AND COMPLICATIONS

### *Treatment*

As stated above, if a patient has been clearly diagnosed with PEComa before surgery, the treatment is not only as simple as surgical resection. First, the study data showed that the risk of metastasis and death from surgical treatment was estimated to be 0.8% (2/247 for metastasis and death, mortality = 0.8%). Progression occurred in 6/35 (21.4%) patients who received conservative treatment[4].

**Conservative treatment:** The basis for choosing conservative treatment is as follows. First, PEComas can be not only single tumors but also manifestations of TSC. TSC is a hereditary disease characterized by seizures, tumor development in the the brain, heart, kidney and skin, and a unique set of neurodevelopmental syndromes known as TSC-associated neurological disease (TAND)[35]. PEComas occur in TSC patients due to biallelic inactivation of TSC2 (more common) or TSC1[36]. The first mutation event (HIT) in TSC2 is a germline mutation, which is the cause of an individual TSC. The second 'HIT' event leads to excessive activation of mTORC1 (a mammalian target of rapamycin complex 1) and promotes tumor development[37,38]. The changes caused by these genes have been proven to be related to the etiology of PEComas. In view of the above findings, inhibitors of the mTOR signaling pathway, such as sirolimus or everolimus, are considered likely to play a role in the treatment of PEComas[24,39]. A study by Martignoni *et al*[24] showed that activated mTORC1 has important functions regardless of whether it is associated with PEComas. In an animal TSC model study before the clinical stage study, the mTOR inhibitor sirolimus showed substantial efficacy[27]. In further experimental studies, Wagner *et al*[40] reported the positive efficacy of the oral mTOR inhibitor sirolimus in the treatment of three patients with malignant PEComas based on changes in tumor imaging data, indicating that this drug may be useful as an immunotherapy for PEComas. Italiano *et al*[41] also reported this. Moreover, in PEComas, which are unresectable in clinical surgery, the use of the mTOR inhibitor sirolimus for neoadjuvant therapy can help the tumor shrink tumors and enable surgical resection[42]. In a recent study, immunohistochemistry and multiple immunofluorescence analyses revealed that HPEComas contain a large number of nontumor cells, mainly lymphocytes and CD68+ macrophages. This phenomenon indicates that HPEComas have a high level of immune cells, which may suggest that the tumor has inert behavior[43]. This provides additional indications for conservative treatment. In summary, conservative treatment and follow-up examination may be effective ways to treat PEComas, especially for patients who are asymptomatic, have small tumors or are considered unsuitable for surgery[6]. Overall, the vast majority of PEComas are benign and tend to grow slowly, while malignant PEComas are extremely rare. Moreover, long-term conservative treatment and follow-up may also have a positive effect or timely effect on the malignant transformation of PEComas at a certain node in the development process. Thus, the survival time of patients should be prolonged. However, additional clinical trials are still needed to confirm these findings.

**Surgical treatment:** The choice of direct surgical treatment is mainly due to the following considerations. First, in patients undergoing surgical treatment, the risk is estimated to be 0.8% (2/247 metastasis and death, mortality rate = 0.8%)[4]. This approach can completely reach the standard of clinical remission. Second, if the preoperative diagnosis of liver PEComas is confirmed by imaging technology or fine needle aspiration biopsy and if the patient has symptoms or may rupture due to a substantial increase in the size of the lesion under continuous observation, surgical resection should be recommended[9]. Furthermore, because the risk of malignant transformation during the development process is unknown, surgical resection should be selected when there is no definite treatment for advanced PEComas[44]. Moreover, Panahova *et al*[45] reported that performing only perforation biopsy may not be sufficient to assess whether a PEComa is a malignant tumor because only surgical resection specimens can reveal the ratio of invasive growth to mitosis. Finally, liver transplantation is the final treatment for unresectable PEComas with large or numerous liver tumors[17,46]. If the patient's tumor cannot be surgically removed, neoadjuvant conversion therapy seems to be a good strategy for treating PEComas that are positive for PET tracers according to imaging, as this approach can transform the tumor and make the patient eligible for surgical treatment[47].

Based on the above analysis, the treatment strategy proposed by Yang *et al*[14] may be appropriate. The authors advocated imaging observation and conservative treatment for patients who: (1) Had a first diagnosis of PEComa; (2) had a lesion size < 5 cm, (3) were expected to have good compliance with follow-up; and (4) did not have viral hepatitis. Because the cumulative estimated increase in the size of these tumors is only 0.77 cm/year, the first surveillance imaging can be performed 1 year after diagnosis, followed by two years of surveillance. When the imaging diagnosis is uncertain, biopsy can be performed. Resection is recommended if the biopsy provides an uncertain diagnosis or if the patient has malignant risk factors such as epithelioid features or high proliferative activity. Other indications for resection include symptoms or invasive growth[4]. In addition, TSC patients may require longer or more frequent monitoring because TSC appears to be a risk factor for progression[4].

### *Complications*

In terms of complications during tumor development, the most common complication of PEComas is malignant behavior, although there is no consensus on what factors constitute invasive or malignant PEComas[3]. At present, imaging evidence of liver PEComa invasion is rare. To date, only 16 patients with liver, omentum, lung or bone metastases have been reported in the literature[14,15,19,30,48-60]. In addition, spontaneous bleeding may also occur in liver AML patients, but the risk of occurrence seems to be lower in liver AML patients than in renal AML patients, possibly because a single vessel is usually involved in the latter and is associated with aneurysms[61]. Arterial embolization is sometimes necessary when spontaneous bleeding occurs[62]. At present, only 8 cases of hepatic angiomyolipoma (HAML) have been reported to cause spontaneous rupture and hemorrhage. The median size of these tumors was 8.5 cm (range: 2.5 cm to 12.5 cm),

and 3 of them were treated with hepatectomy after arterial embolization; these patients were formally diagnosed with HAML[39]. This may indicate that spontaneous bleeding usually occurs from larger lesions[28].

In conclusion, Kou *et al*'s concern about the preoperative misdiagnosis of liver PEComas is entirely reasonable and necessary[1]. The preoperative diagnosis of HPEComas is very important. Accurate diagnosis can change the treatment and prognosis of patients. Imaging and serological tests are the first step, followed by biopsy. However, we also need to point out that the clinical reality is often more complex than theoretical accounts, as in the three cases reported by Kou *et al*[1]. Although all the patients were subjected to ultrasound, three-phase enhanced tomography, enhanced MRI, and intraoperative frozen pathology, the results still suggested HCC. This suggests that clinicians, imaging experts, and surgical pathologists must be aware of other rare disease entities that may be involved in the diagnosis of liver tumors and should not directly ignore suspicious signs that may point to other diagnoses, such as normal serum tumor markers. Maintaining a skeptical attitude toward the diagnostic results and carefully verifying them are the keys to revealing additional unknown clinical problems. In 2002, WHO formally defined "PEComas" as mesenchymal tumors composed of histologically and immunohistochemically unique PECs, and PEComas include many different clinicopathological entities. Among them are angiomyolipoma, lymphangioma, lymphangioleiomyomatosis, clear cell tumor sugar and tumor types not otherwise specified[2]. Although PEComas and AMLs are theoretically subordinate, many clinical experts believe that the two are actually different manifestations of the same disease[3]. Therefore, in the following, we will discuss AMLs and PEComas as unified concepts and no longer make a special distinction.

**Definition and tumor properties:** It is currently clear that PEComas are mesenchymal tissue-derived tumors that are usually composed of blood vessels, smooth muscle and adipocytes[4]. However, the proportions of these tissue components tissue components differ among patients[5]. These patients often complain of abdominal discomfort but do not present accompanying abnormal serological test results[6-8]. At present, the pathogenesis of this tumor has not been elucidated[5,6]. Although more than 50% of renal AMLs are associated with TSC, it is estimated that only 5%-15% of patients with solitary liver tumors have such a link[4]. The vast majority of PEComas are benign, and malignant forms are extremely rare[9]. In view of the relatively few reports on malignant PEComas, a clear malignant standard has not yet been established[9]. However, even if PEComas are identified as benign, their boundary with malignant tumors is not very clear. First, cytological atypia, a characteristic of malignant tumors, is present in benign liver PEComas[10], while the consistent characteristic of malignant liver PEComas is considered to be coagulative necrosis[9]. Therefore, only when the tumor has necrosis, a large mass (> 10 cm), CD117 negativity, invasive behavior or other clinical evidence may it be considered a malignant liver PEComa[10]. Second, it has been reported that benign PEComas may even undergo malignant transformation during development, eventually displaying sarcomatoid or cancer-like characteristics[11]. Moreover, some patients have also been found to have advanced metastasis many years after the diagnosis of primary benign tumors[11]. Furthermore, in a recently proposed classification system, Folpe *et al*[12] divided PEComas into benign, UMP and malignant tumor categories. We think that this classification may be appropriate because it identifies a variety of tumor behaviors shown during the development of PEComas, clearly specifying UMP and recognizing that malignant tumors may occur given malignant behaviors or features. When there are multiple malignant changes, the tumor can be defined as malignant. Finally, regarding the factors that lead to the malignant transformation of PEComas, several hypotheses have been proposed. It has been reported that malignant behavior occurs mainly in epithelioid PEComas and can be observed in the early stage of tumorigenesis[13]. The diagnosis of epithelioid PEComa in a patient by clinical examination indicates that there is a greater possibility of malignant transformation, and a treatment strategy for malignant tumors should be provided.

**Preoperative diagnosis:** As discussed by Kou *et al*[1], PEComas have a very high preoperative misdiagnosis rate. According to Yang *et al*[14], Zeng *et al*[15] and Jung *et al*[16], only 18%-26% of patients with histopathologically confirmed PEComas were correctly diagnosed before surgery. This phenomenon may be due to many factors.

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## FOOTNOTES

**Author contributions:** Yan S and Lu JJ were responsible for the revision of the manuscript for important intellectual content; Wu JZ and

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**Country/Territory of origin:** China

**ORCID number:** Shuai Yan 0009-0000-3691-2077; Jia-Jie Lu 0009-0007-1789-8004; Lin Chen 0000-0002-1108-4735; Wei-Hua Cai 0009-0002-9550-2558; Jin-Zhu Wu 0000-0002-7295-2030.

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