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Contents

Weekly Volume 29 Number 13 April 7, 2023

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Case Control Study

Retrospective Study

Observational Study

Randomized Clinical Trial

SYSTEMATIC REVIEWS

ABOUT COVER

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REVIEW

Application of nanotechnology in reversing therapeutic resistance and controlling metastasis of colorectal cancer

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Abstract

Colorectal cancer (CRC) is the most common digestive malignancy across the world. Its first-line treatments applied in the routine clinical setting include surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. However, resistance to therapy has been identified as the major clinical challenge that fails the treatment method, leading to recurrence and distant metastasis. An increasing number of studies have been attempting to explore the underlying mechanisms of the resistance of CRC cells to different therapies, which can be summarized into two aspects: (1) The intrinsic characters and adapted alterations of CRC cells before and during treatment that regulate the drug metabolism, drug transport, drug target, and the activation of signaling pathways; and (2) the suppressive features of the tumor microenvironment (TME). To combat the issue of therapeutic resistance, effective strategies are warranted with a focus on the restoration of CRC cells' sensitivity to specific treatments as well as reprogramming impressive TME into stimulatory conditions. To date, nanotechnology seems promising with scope for improvement of drug mobility, treatment efficacy, and reduction of systemic toxicity. The instinctive advantages offered by nanomaterials enable the diversity of loading cargoes to increase drug concentration and targeting specificity, as well as offer a platform for trying the combination of different treatments to eventually prevent tumor recurrence, metastasis, and reversion of therapy resistance. The present review intends to summarize the known mechanisms of CRC resistance to chemotherapy, radiotherapy, immunotherapy, and targeted therapy, as well as the process of metastasis. We have also emphasized the recent application of nanomaterials in combating therapeutic resistance and preventing metastasis either by combining

with other treatment approaches or alone. In summary, nanomedicine is an emerging technology with potential for CRC treatment; hence, efforts should be devoted to targeting cancer cells for the restoration of therapeutic sensitivity as well as reprogramming the TME. It is believed that the combined strategy will be beneficial to achieve synergistic outcomes contributing to control and management of CRC in the future.

Key Words: Colorectal cancer; Therapeutic resistance; Nanotechnology; Drug delivery system; Combined treatment

Core Tip: Mechanisms of Colorectal cancer (CRC) cell resistance can be attributed to tumoral and environmental factors, the former of which includes gene expression alteration, signaling pathway activation, metabolic rewiring, and the later refers to complicated adaptions regarding cancer associated fibroblast, immune cells, hypoxic conditions. Taking efforts to conquer therapeutic resistance is imperative to improve CRC patients` survival. Nanotechnology possesses distinct advantages to increase specificity of treatment and realize codelivery of multiple drugs, which facilitates to restore sensitivity to antitumor therapy, and modulate suppressive tumor microenvironment to stimulatory environment.

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INTRODUCTION

Colorectal cancer (CRC) is the most common malignancy of the digestive tract, accounting for the death of 0.9 million people across the world in 2020 alone[1]. Surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are among the present first-line strategies applied to treat CRC patients and control their recurrence. Surgery is accepted as the primary treatment for CRC, especially for early stage CRC, as it eradiates the local lesions thoroughly in a timely manner[2]. Five-Flurouracil (5-FU)- and oxaliplatin-based chemotherapy contribute to the control of local recurrence and metastasis of CRC after surgery. As for advanced CRC, radiotherapy and neoadjuvant chemotherapy are important modalities that have been reported to contribute to prolonging the patients' survival[3,4]. In addition, cetuximab and immunocheckpoint inhibitor (ICI)-based therapy can be administrated to EGFR-overexpressed CRC or microsatellite instability (MSI)-subtype CRC so as to achieve greater survival benefits[5, 6]. However, despite the availability of comprehensive treatment against CRC, resistance to therapy and metastasis continue to be reported during or after the treatment, often resulting in relapse or treatment failure.

Chemotherapy resistance is a common cause of CRC treatment failure, and the intrinsic and acquired resistance of tumor cells have been implicated as the major causes[7]. Aberrant metabolism and change in the drug targets have generally been reported to be responsible for the 5-FU resistance of CRC cells developed during the treatment duration[8,9]. The development of resistance to radiotherapy is usually related to hypoxia and the adaption of cancer stem cells (CSC) that play a pivotal role in the regulation of survival and proliferation of CRC[10]. In addition, the tumor microenvironment (TME) is involved in the revolution and development of tumor cells to resist attacks from drugs and immune cells[11]. Many efforts have been made toward enhancing the treatment efficacy after resistance is developed, such as by increasing the drug dosage or combining multiple treatments. However, the improvements in response and the overall survival rate remain unsatisfactory due to the issues of increased toxicity and poor tolerance. Consequently, more effective drug-delivery methods and therapeutic strategies are urgently warranted to overcome the barrier of the suppressive microenvironment as well as to restore the sensitivity of CRC cells to the prescribed treatment.

Thanks to the unique advantages offered by nanotechnology, it has drawn the great attention of researchers and clinicians in their quest to conquer the issue of treatment resistance of CRC with its valuable potential. Drug-delivery systems based on nanomaterials can simultaneously carry a variety of components and increase the selectivity of the systemic treatment and the local drug concentration through active and passive targeting effects[12-14]. This approach not only delivers cytotoxic drugs but also delivers nucleic acid molecules, thereby realizing the combined application of chemotherapy,

immunotherapy, gene therapy, and other treatments[15-17]. In addition, several nanomaterials possess anticancer potential or can hence be utilized as radiotherapy sensitizers and they generate a photothermal or photodynamic effect that can facilitate tumor control[18,19]. When used either intraoperatively or postoperatively, nanomaterial-based treatment can be applied to remodel the local microenvironment and activate the innate and adaptive immune responses toward controlling tumor growth and metastasis, which, in turn, generate immune-memory effects called immunomodulation[20-22]. Collectively, nanotechnology is expected to solve the treatment resistance of CRC as a valuable alteration to realize the effective control of tumor recurrence and metastasis. In this review, we have illustrated the mechanisms of therapeutic resistance of CRC cells to various treatments, including both tumoral and environmental factors (Figure 1). Meanwhile, recent advancements in nanotechnology utilized in CRC treatment aimed at restoring tumor sensitivity and reversing resistance will be further introduced, along with the challenges and perspectives for nanotechnology-based CRC treatment.

MECHANISMS OF THERAPEUTIC RESISTANCE AND METASTASIS

Chemotherapy resistance

Fluorouracil, oxaliplatin, and irinotecan are the three main reagents used for CRC adjuvant chemotherapy, which exert antitumor effects through interaction with DNA. However, during the treatment, tumor cells adapt several mechanisms to evade the attack of the treatment agent. Generally, the tumoral mechanism of developing resistance can be categorized into 4 aspects: alterations in the pharmacokinetics and metabolism, transport process, and targets of chemotherapeutics as well as aberrant activation of oncogenic/bypass signaling (Figure 1).

In the case of pharmacokinetics and drug metabolism, reduced concentrations, decreased activation, and increased inactivation of drugs in tumor site, all contribute to the adaption of resistance. Efforts have been focused on investigating the importance of pharmacogenomics on intrinsic and acquired resistance in CRC. Polymorphism of genes encoding critical enzymes during drug absorption, distribution, metabolism and detoxication, has been identified which will facilitate targeted personalized therapy, such as UDP-glucuronosyltransferases (UGT1As) and glutathione-S-transferase (GSTs)[23]. The transformation of capecitabine (CAP) to 5-FU is essential for direct cytotoxicity, which is mediated by thymine phosphorylase (TP)[24]. According to a past report, tumor cells apply epigenetic alterations to downregulate the TP expression, which leads to the development of drug resistance to CAP, albeit it can be reversed by DNA methyltransferase[25]. Similarly, changes in the activity of the key enzymes of 5-FU and irinotecan were evaluated by CRC cells affecting drug sensitivity[8,9,26,27]. Moreover, the inactivation of drug metabolites facilitates the development of drug resistance by CRC cells. Considering that dihydropyrimidine dehydrogenase (DPD) catabolizes 5-FU into an inactive product, the investigation of CRC patients revealed that a high level of DPD expression was significantly related to the resistance to 5-FU[28]. Irinotecan and its active form SN38 can be converted into an inactive form by several enzymes, such as cytochrome P450 (CYP) 3A4 and uridine diphosphate glucuronosyltransferase 1A, and therapeutic strategies targeting these enzymes have been proposed to effectively reverse irinotecan resistance[29,30].

Changes in drug transport are another important factor for drug resistance. Membrane transport pumps can transport chemotherapeutic drugs or molecular targeted drugs out of the cells, thereby reducing the intracellular concentrations and leading to the development of multidrug resistance (MDR). Membrane transporters mainly include 2 families: ATP binding cassette (ABC) and solute carrier transporters. ABC transporters are the most famous and the main membrane transporter for MDR, executing an important role in CRC resistance to chemotherapy. P-glycoprotein (P-gp) is the first identified ABC superfamily member, and its upregulated expression has been implicated in the development of MDR leading to chemotherapy failure in CRC[31,32]. To date, several preclinical studies have demonstrated that natural inhibitors targeting ABCs or antisense oligonucleotide and ncRNAs aiming to downregulate the P-gp expression possess the therapeutic potential to reverse the resistance of CRC cells to oxaliplatin and 5-FU[33-36].

Alterations in the drug targets play a role in mediating the generation of drug resistance. Thymidylate synthase (TS) is the main target of 5-FU in destroying DNA replication, and its expression plays a crucial role in the development of CRC resistance to 5-FU[28]. Efforts to interfere with 5-FU-mediated TS inhibition are expected to disrupt the development of drug resistance. Folic acid (FA) enhances the cytotoxicity of 5-FU through the inhibition of TS both *in vitro* and *in vivo*[37,38]. Topoisomerase 1 (TOP-1) is the main target of irinotecan, and its mutation or downregulation is responsible for CRC resistance, as reduced TOP-1 binding to DNA also contributes to irinotecan resistance[27,39]. Past evidence suggests that Y-box binding protein 1 (YB-1) can improve the sensitivity of irinotecan through its direct interaction with TOP-1 toward promoting intracellular TOP-1 activity [40].

As for oncogenic/bypass signaling, it has been demonstrated that the sustained activation of Wnt/β-catenin, JAK/STAT, and TGF-β contributes to drug resistance of CRC. Wnt/β-catenin signaling is a well-known evolutionarily conserved pathway endowing tumor cells with sustained growth and is

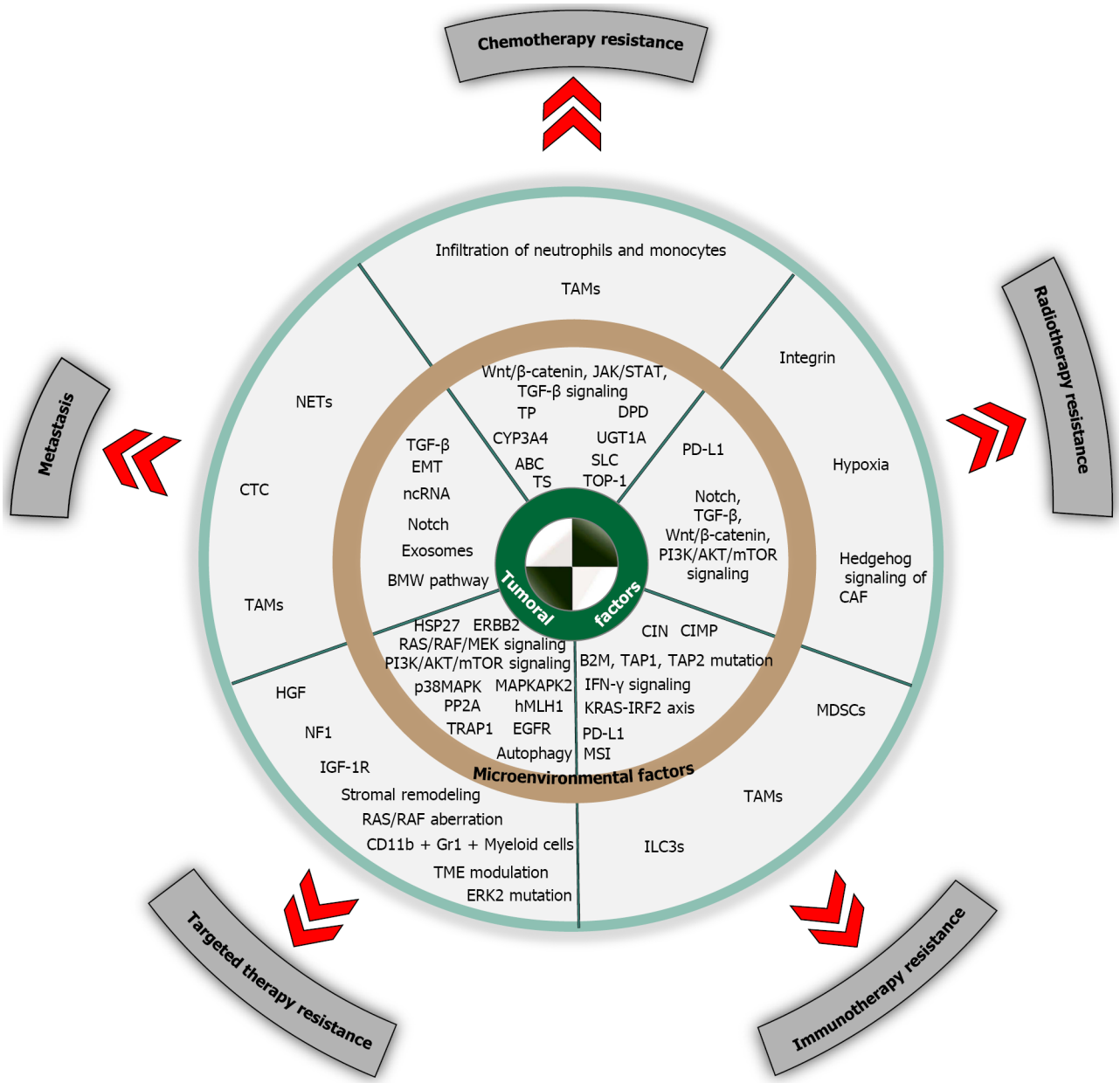

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Figure 1 Schematic illustration of mechanisms of colorectal cancer cell resistance to chemotherapy, radiotherapy, immunotherapy, targeted therapy, and metastasis. ABC: ATP binding cassette; B2M: β2-microglobulin; CAFs: Cancer associated fibroblasts; CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; CTC: Circulating tumor cell; CYP3A4: Cytochrome P450 3A4; DPD: Dihydropyrimidine dehydrogenase; EGFR: Epidermal growth factor receptor; EMT: Epithelial-mesenchymal transition; ERBB2: Erb-b2 receptor tyrosine kinase 2; ERK2: Extracellular regulated protein kinases; HGF: Hepatocyte growth factor; hMLH1: Human MutL homolog 1; HSP27: Heat shock protein 27; IGF-1R: Insulin-like growth factor 1 receptor; ILC3s: Group 3 innate lymphoid cells; MAPKAPK2: Mitogen-activated protein; MDSCs: Myeloid-derived suppressive cells; MSI: Microsatellite instability; NETs: Neutrophil extracellular traps; NF1: Neurofibromin 1; p38MAPK: p38 mitogen-activated protein kinase; PP2A: Protein phosphatase 2A; SLC: Solute carrier; TAP1: Transporter 1; TAP2: Transporter 2; TAMs: Tumor associated macrophages; TME: Tumor microenvironment; TOP-1: Topoisomerase 1; TP: Thymine phosphorylase; TRAP1: TNF receptor associated protein 1; TS: Thymidylate synthase; UGT1A: Uridine diphosphate glucuronosyltransferase 1A.

responsible for CRC resistance to chemotherapy[41]. The inhibition of the Wnt/β-catenin pathway can restore the CRC sensitivity to 5-FU, thus exhibiting potential therapeutic potentials[42]. JAK/STAT signaling has been reported to regulate cell survival, proliferation, apoptosis, and differentiation, which is associated with CRC cells resistant to chemotherapy and immunotherapy[43-45]. TGF-β signaling also participates in the development of CRC resistance to chemotherapy through the upregulation of mesenchymal maker expression and inducement of MEK/ERK activation[46,47]. Targeted therapy against TGF-β thus presents promising value toward reducing the growth of chemo-resistant CRC cells and facilitating tumor control[48]. Last but not least, PI3K/AKT/mTOR pathway is well-accepted to be involved in tumorigenesis and development of CRC, which also contributes a lot to development of chemotherapy resistance[49]. Recent study identified miRNAs responsible for 5-FU resistance of CRC

cell lines through regulating activity of PI3K/AKT pathway[50]. Moreover, PI3K/AKT/HIF-1α and AKT/GSK-3β/Snail signals also contribute to drug resistance *via* regulation of glycolysis and stem cell maintenance, respectively[51,52].

An increasing body of evidence has demonstrated crosstalk between tumor cells and the microenvironment, and the impact of the TME on chemotherapeutic efficacy suggests another mechanism for drug resistance. For instance, tumor-associated macrophages (TAMs) facilitate tumor growth, metastasis, and angiogenesis, while suppressing the anticancer immune response, regulating metabolism, and even influencing the microbiota[53]. Recently, multi-omics research utilized a large sample to reveal increased infiltration of neutrophils and monocytes in chemotherapy-resistant CRC tissues, which has been associated with poor outcomes[54]. Meanwhile, another research by Gupta *et al*[55] investigated metastatic CRC patients who received neoadjuvant chemotherapy. The results revealed that the TME was significantly altered after chemotherapy when neoadjuvant chemotherapy increased the levels of T cells, especially $CD8^+$ T cells. Furthermore, bioinformatic studies focused on the prediction of sensitivity to chemotherapy and other therapies analyzing features of the TME and other pathological conditions have provided some promising results[56,57].

Epithelial-mesenchymal transition (EMT) also represents another form of interaction between tumor and microenvironment, especially during development of chemoresistance. Most tumor cells with the capacity of chemoresistance show a strong mesenchymal phenotype. EMT driven by miRNAs, oncogenes, protein kinases, and other factors facilitates to generation of drug resistance. Upregulated expression of DPYD, TYMS, ERCC1, and GSTP1 was associated with EMT, which eventually leads to 5-FU and oxaliplatin resistance of CRC cells[58,59]. DSTYK, a novel protein kinase, also contributes to chemoresistance in CRC cells by activating TGF-β induced EMT, and knockout of DSTYK hinders tumor growth *in vivo*[60]. Recently, proven by Zhu *et al*[61], activation of SOX2-β-catenin/Beclin1/autophagy signaling generated promoting effects on EMT, stemness and chemoresistance in CRC.

In addition to EMT, extracellular vesicles (EVs) are considered as a major pathway for cell-cell communication *via* transfer of their cargo, which ranges from proteins, lipids, miRNAs, and circRNAs. Due to the diversity of EVs cargo, it plays essential regulatory role during chemoresistance[62]. So far, compelling evidence indicated miRNAs transferred by EVs can be utilized as predictive marker of chemoresistance, and miR-21 was identified as the most common EV-associated miRNA relating to chemoresistance in CRC[63]. It was indicated that CRC exosomes are able to activate Wnt/β-catenin pathway through promoting the stabilization and nuclear translocation of β-catenin leading to 5-FU and oxaliplatin resistance[64]. CircRNA-122 delivered by EVs originated from chemoresistant CRC cells, played robust promoting effect on glycolysis which reduced drug susceptibility in chemosensitive cells [65]. In addition to harboring regulatory proteins, miRNAs, and circRNAs, drug efflux pumps (such as P-gp 1) and even cytotoxic drugs can also be transported by EVs to sequester drugs and negate drug effect on tumor cells[66-69]. Moreover, EVs generated from TME are also responsible for generation of chemoresistance of CRC. In a pre-clinical research, cancer-associated fibroblast (CAF)-derived EVs showed remarkable inhibitory effect on anti-cancer activity of oxaliplatin[70].

Radiotherapy resistance

Radiotherapy is considered another important adjuvant treatment to treat CRC, especially for advanced rectal cancer (as recommended by the NCCN guideline). However, resistance to radiotherapy is prevalent, which can be attributed to two main factors: tumor heterogeneity and the complexity of TME. Tumor heterogeneity is the most important reason for radiotherapy resistance, as supported by the fact that the equal effect of radiotherapy is not achieved in all tumor subpopulations. Among them, CSCs have been implicated as the most responsible factor for resistance to several therapies including radiotherapy[71-73]. Resistance to radiotherapy of CSCs can be categorized as intrinsic and acquired resistances, the latter of which is the adaptive response caused by radiotherapy itself. Owing to the distinct quiescent state and the plasticity of CSCs, they can survive stress, chemotherapy, and radiotherapy, which is referred to as intrinsic resistance[74]. Past studies have demonstrated that the activation of several signaling pathways contributes to CSC radio resistance, which includes Wnt/β-catenin, Notch signaling, TGF-β, Hedgehog, and PI3K/AKT/mTOR signaling. Wnt signaling increases the active levels of β-catenin post-radiation, leading to the development of resistance, which plays a role in the dedifferentiation of CSCs by regulating the SOX2 expression[75-78]. The Notch pathway can be activated *via* radiation and initiation of gene transcription, leading to an increased proportion of cells in the S phase[78]. The Hedgehog pathway is involved in the reprogramming of CAFs and is also responsible for the production of a supportive condition for the maintenance of CSCs stemness[79]. In addition to the alterations of the signaling pathways, tumor cells, especially CSCs, take adaptions of apoptosis, cell cycle, and EMT to survive radiation[74]. The cell-cycle progression can be blocked or slowed by irradiation that induces the redistribution of cells in the S phase, thereby contributing to radio resistance[80]. Researchers have found that the EMT triggered by irradiation may be related to the intravascular behavior of tumor cells forming the circulating tumor cells (CTCs) that are deemed responsible for the recurrence and metastasis[81,82].

Currently, a plethora of evidence indicates the significance of TME involved in radio resistance of solid tumors[74,83,84]. Concerns regarding the TME on radioresistance are based on the observation of COMMA-D cells, which are rarely tumorigenic and present with several features of normal mammary

epithelial cells. In a mouse model, the implantation of unirradiated COMMA-D cells into the mammary fat pads of irradiated hosts eventually formed large tumors, indicating the pivotal role of the TME in the determination of the radiotherapy outcome[85]. Generally, the effects of radiotherapy on the TME can be categorized into 3 forms: Vasculature, stroma, and immune system[84]. Irradiation damages the vasculature within tumors by causing the dysfunction of endothelial cells. The main consequence of radiation is hypoxia induced by increased permeability and detachment of the endothelial cells from the basement membrane as well as apoptosis[86,87]. To date, hypoxia has been recognized as one of the most critical reasons for radio resistance of tumors, and several strategies have been devised to reverse this resistance by overcoming the hypoxic environment. Ritter *et al*[88] reported that exposure to acute and adaptation to chronic hypoxia alters the balance of Bcl-2 family proteins in favor of anti-apoptotic family members, thus attenuating the success of radiotherapy[88]. Moreover, exposure to hypoxia can activate oxygen-sensitive signaling cascades leading to metabolic adaptation and increasing cell death threshold, which accounts for a worse response to radiotherapy[89].

Regarding the impact on the stroma, radiotherapy induces significant activation of CAFs. The upregulation of integrin by CAFs participates in radio resistance through the interactions among cancer cells, stroma cells, and the environment[90,91]. Finally, the immune effect of radiotherapy has been well studied considering that induction of immunogenic cell death (ICD) after irradiation can, in turn, induce anticancer response through the release of neoantigens[92]. The resultant change in inflammation can be caused by the alterations in the cytokine signaling pathways in the TME under hypoxia-created irradiation, which recruits both immunostimulatory and immunosuppressive cells to locate the tumor site[93,94]. Moreover, the exhaustion of $CD8^+$ T-cell induced by radiotherapy is another radio-resistant mechanism that is supported by the upregulation of PD-L1 on tumor cells after radiation therapy[95-97]. In such a case, the final immunomodulation of radiation therapy can be stimulatory or suppressive, which offers therapeutic challenges to confer the synergistic potential of radiation therapy combined with immunotherapy[84].

Immunotherapy resistance

Immunotherapy has long been recognized as an effective modality to treat multiple malignancies, albeit its efficacies on CRC remain confined to only a few subtypes. Generally, resistance to immunotherapy can be attributed to tumoral and microenvironmental factors. With regard to the tumor factors, it is well-acknowledged that CRCs with microsatellite stability is characterized by considerably low tumor mutation burden (TMB) and less immune infiltration when compared with CRCs exhibiting MSI. FDA-approved immunotherapy suggested certain outcomes in CRC patients with MSI metastatic disease[98,99]. In addition to MSI, two other pathways have been reported to be responsible for genomic instability, including chromosomal instability (CIN) and the CpG island methylator phenotype, whose identification can facilitate the stratification of immunotherapy-sensitive patients. In theory, the response to ICI is expected in all MSI tumors; however, a certain proportion of patients continues to experience progression due to several evading mechanisms in real clinical practice. Whether intrinsic or adaptive, the specific evading mechanism is not fully understood at present, although changes in some of the signaling pathways and gene expression have been reported[37,100,101].

The main mechanism concerns impaired antigen presentation or altered expression of HLA complex. Mutations of B2M [β2-microglobulin (B2M), or HLA class I heavy chain] in CRC have been reported to be significantly associated with the MSI phenotype but not MSS tumors[102,103]. Moreover, CRC patients with an MSI phenotype and harboring B2M mutations revealed resistance to anti-PD-1 mAb treatment[104]. B2M-mutant MSI tumors showed higher infiltration of PD-1+ T cells relative to B2M-wild-type MSI tumors, indicating the loss of HLA-I expression mediated by B2M mutation[105]. Moreover, the mutations of genes involved in the antigen presentation process, such as TAP1 and TAP2, have been related to the number of TILs in a tumor[106]. Other factors, including mutations in the HLA peptide-binding area, HLA class I transactivator NLR5, and RFX5, have also been cited as responsible for the altered function or expression of HLA that results in the development of immunotherapeutic resistance[101,106,107]. As for the signaling pathway, IFN-γ signaling plays an essential role in immunotherapy. The mutations of *JAK1* and *JAK2*-the downstream kinases of IFN-γ signaling-are associated with an inherent resistant to anti-PD-1 mAb treatment of CRC[45]. Recently, the KRAS-IRF2 axis was illustrated to be responsible for the direct regulate of the CXCL3 expression, which bound to CXCR2 on the surface of myeloid-derived suppressive cells (MDSCs) and promotes the migration of MDSCs to TME, resulting in the development of immunotherapeutic resistance[108].

On the other hand, TME is significantly related to resistance to immunotherapy. Most importantly, the density, function, and site of immune cells' infiltration in tumors are critical to the efficacy of the applied immunotherapy[109,110]. In such a situation, immunoscore is recommended for the evaluation of the immune contexture within a tumor. A score of 0-4 indicates a significant association with the prognosis of CRC patients[111]. Specifically, the expression of PD-L1 has been associated with a higher immunoscore[112,113]. Furthermore, researchers have suggested a novel CRC molecular subtyping system, and four consensus molecular subtypes (CMS) have fully illustrated CRC with distinct molecular and immune features[114]. According to the CMS subtyping, immunotherapeutic efficacy can be successfully predicted by different TMB and immunoscore, which is believed to be superior to those of conventional UICC-TNM staging[106,114-118]. Among the infiltrated immune cells, MDSCs and

TAMs, which are referred to as immunosuppressive myeloid cells, are the major suppressive factors that downregulate the antitumor effect of $CD8^+$T cells. Recently, researchers revealed a novel mechanism responsible for the regulation of MDSCs function through prostaglandin E2 receptor 4 (EP4), and they developed a selective antagonist TP-16 as a potential target for the enhancement of immunotherapy for CRC[119]. Furthermore, the microbiota has also been implicated in the development of immunotherapeutic resistance of CRC[120]. Group 3 innate lymphoid cells (ILC3s) was recently reported to be associated with the alterations in microbiota in human along with the indications of dysfunctional adaptive immunity and immunotherapy resistance[121].

Additionally, many studies have linked tumor EMT phenotype with immunosuppression status through regulating PD-L1 expression, which lead to resistance to therapies. Evidence from a former study has revealed the link between EMT and $CD8^+$T cell immunosuppression. MiR-200, a cell-autonomous suppressor of EMT, regulates expression of PD-L1, but suppressed by EMT activator ZEB1 [122]. Snail, a transcription factor of EMT, was shown to promote tumor growth *in vivo* and confer resistance against T cell-mediated lysis through the induction of autophagy[123,124].

Targeted therapy resistance

In addition to chemotherapy, radiotherapy, and immunotherapy, specific antibodies and small inhibitors therapy targeting cancer-associated receptors or signaling pathways represent another antitumor strategy toward control CRC. There are four main types of targeted therapy applicable in treating metastatic CRC: Monoclonal antibodies targeting EGFR, inhibitors of VEGF, small chemicals targeting intracellular kinases of several signaling pathways, and a recently approved small molecular BRAF inhibitor[125]. Among these, anti-EGFR therapy is widely used to manage KRAS wild-type CRC, including cetuximab and panizumab, the resistance to which will be mainly illustrated in this subsection. Reportedly, anti-EGFR therapy has been effective in 50% of CRC patients showing KRAS wild-type, but only 6% in those showing KRAS-mutant tumor[126,127]. In a clinical setting, patients usually develop resistance to anti-EGFR therapy after 3-12 mo of initiating the treatment[128,129]. Furthermore, it has been estimated that 80% of mCRC patients develop resistance to anti-EGFR regimens[130].

The mechanism of resistance to anti-EGFR therapy can be categorized into 2 types: Tumoral and environmental. Alterations of the EGFR ligands and EGFR significantly contribute to treatment resistance. The mutation of the EGFR kinase domain (V843 I) and the methylation of R198/R200 have been correlated with disease progression in patients receiving cetuximab[131]. Sequence changes in the extracellular domains of EGFR have also been implicated in conferring resistance to anti-EGFR treatment through the prevention of mAb binding[132-134]. The activation of compensatory feedback loop signaling, including RAS/RAF/MEK and PI3K/AKT/mTOR pathway, is also known to contribute to drug resistance[135]. Mutations, amplification, and the loss of genes, as well as aberrant phosphorylation of these pathways, are all important factors in the primary and secondary resistances to anti-EGFR therapy[136,137]. For instance, mutation of BRAF has been identified as the major marker of poor prognosis of mCRC after anti-EGFR treatment[138-140]. As a dominant downstream factor of EGFR signaling, mutations and abnormal activation of the PI3K/AKT/mTOR pathway is responsible for resistance to anti-EGFR[136,141,142]. Genomic stability is not only related to immunotherapy efficacy but also the development of anti-EGFR resistance. Moreover, it has been reported that hMLH1 deficiency generated resistance to cetuximab through the increased expression of ERBB2 and downstream PI3K/AKT signaling[143]. On the other hand, EGFR-targeted treatment has been reported to increase the levels of immune cells' infiltration and PD-L1 expression, suggesting the potential of treatment combination with immunotherapy to combat the resistance of CRC[135].

CSCs are considered the predominant reason for treatment resistance. After targeted therapy, CSCs aberrantly activate the substitute receptor tyrosine kinase, leading to the development of resistance to anti-EGFR therapy[144]. Moreover, they activate anti-apoptotic signaling to generate resistance to bevacizumab, such as PP2A, p38MAPK, MAPKAPK2, and Hsp27[145,146]. During an antitumor treatment, cancer cells takeover metabolic remodeling to sustain survival, which contributes to the development of resistance to targeted therapy. It has been reported that elevated levels of glycolysis mediated by TRAP1 is related to resistance to anti-EGFR therapy and that fatty acid metabolism demonstrated certain anti-apoptotic effect during the cetuximab treatment[147,148]. Furthermore, autophagy is responsible for developing resistance to the anti-EGFR regiment[149,150].

In terms of the environmental factors, TME plasticity plays a significant role in modulating resistance to targeted therapy[149]. The dysfunction of immune cells, altered infiltration of CAFs, and angiogenesis all have an impact on the efficacy of anti-EGFR treatment[151]. Hepatocyte growth factor (HGF) secreted by CAFs binds MET receptor and activates MAPK and AKT signaling, thereby inducing resistance of CRC cells to cetuximab[144]. Moreover, Woolston *et al*[129] applied genomic and transcriptomic analyses to evaluate primary and secondary resistances of CRC during anti-EGFR treatment and revealed that NF1 and non-canonical RAS/RAF aberrations are associated with primary resistance. Importantly, stromal remodeling mediated by CAFs has been indicated as a non-genetic mechanism of cetuximab resistance[152]. In addition, treatment-induced senescence is believed to exert a pro-tumorigenic effect, leading to recurrence and progression, which can be attributed to the secretion of several factors and genetic mutations[153,154].

To compensate for the prevalent resistance to anti-EGFR regimens, the combined application of other targeted therapies has been proposed and tested in preclinical and clinical studies. Although several agent-targeted RAS mutations have been approved in the treatment of CRC[155-157], the resultant response is not sufficiently long for most patients, and the underlying mechanism included the upregulation of some compensatory signaling events such as the TME modulation and recruitment of CD11b+ Gr1+ myeloid cells[158]. Anti-IGF-1R treatment has been recognized as a targeted strategy in the treatment of mCRC, the resistance to which has also been encountered in clinical studies. The underlying mechanism can be attributed to the nuclear location of IGF-1R during the treatment, which leads to the development of resistance to anti-IGF-1R therapy as well as chemotherapy[159]. In a study of 47 human CRC cell lines, the authors observed a synergistic inhibition effect with the combination of MEK and PI3K inhibitors, although primary and secondary resistances were observed and the mutation of ERK2 was recommended as the driver of resistance to the mono- or combined regimens[160].

Since approved by FDA in 2004, Bevacizumab (Avastin) and other VEGF pathway inhibitors have been utilized for cancer therapy, which improved survival in most cancer patients but some still have limited or no benefit from them. The primary and acquired resistance can be attributed mainly to activation of alternative angiogenesis other than VEGF pathway, including angiopoietin (Ang2), bombina variegate (Bv8), FGF, *etc*[161,162]. Additionally, other factors also play important role in development of anti-VEGF therapy resistance. Inflammation was tested to affect responsiveness to anti-VEGF in a mouse model of chemical induced colitis, which revealed Bv8/PROK2 expressed by tumor-infiltrating neutrophils related to anti-VEGF resistance[163]. Specifically, patients with $CD177^{+}$ neutrophil infiltration in tumor tissues had poor survival than those with $CD177^{-}$ infiltrates[164]. Besides, HOXB9 and extracellular matrix (ECM) remodeling induced by anti-VEGF were responsible for development of anti-VEGF resistance[165,166]. Park *et al*[167] found that nearly all of the cetuximab-resistant colorectal cancer cells showed a higher EMT signature, showing increased EMT markers such as SNAI2.

Metastasis

Metastatic events are the major cause of CRC patients' death, with the liver being the most common site of metastasis[168-170]. The lung, distant lymphatic nodes, and peritoneum are the secondary metastatic sites of CRC[168]. In the 1880s, the classic concept of "seed (tumor cells) and soil (specific organs) was proposed by Paget, which illustrated the basic metastatic mechanism for tumors[171]. To date, several excellent reviews have detailed the specific intravasation and extravasation procedures of CRC cells during metastasis[172-176]. For instance, liver metastasis is recognized as multiple factors- and processes-involved complex biological procedures. First, a certain subtype of CRC cells is endowed with the invasion capability, and they migrate to the ECM through the EMT, followed by infiltration into the surrounding tissues, after which they enter and survive in circulation, eventually colonizing and forming a secondary tumor in distant organs *via* extravasation[172]. Moreover, a metastatic mouse model explored by Enquist *et al*[177] suggested that the direct spread of CRC cells through the blood to the liver is a possible dissemination route without any prior lymph-node involvement; this information has the potential to facilitate future drug development targeting metastasis[177,178].

As far as the intrinsic factors are concerned, several molecular mechanisms have been deemed responsible for CRC metastasis, including non-coding RNAs (ncRNAs), Notch, TGF-β, and exosomes [172]. Among these, TGF-β is widely accepted as the most important signaling pathway related to metastasis. It was recently reported that an increased level of TGF-β in the TME promoted T-cell exclusion and blocked the acquisition of the T_H1-effector phenotype, while the blockage of TGF-β signaling rendered CRC susceptible to immunotherapy[179]. To induce the EMT process, TGF-β downregulated the expression of E-cadherin and upregulated the Vimentin expression in CRCs in order to promote invasion and migration[180]. Consequently, TGF-β signaling may serve as a vulnerable factor of CRC metastasis that can be exploited for targeted therapy in a clinical setting.

In addition to the EMT process mentioned earlier, recent studies have indicated a cell death process-dependent pathway for metastasis[181]. Among the several reversible cell death forms (such as apoptosis, necroptosis, ferroptosis, pyroptosis, and NETosis), the Blebbishield emergency program is considered a prototype for cell death-driven metastatic pathway[176]. The Blebbishield metastatic-witch pathway is characterized by the formation of apoptotic blebbing that is reconstructed into blebbishields and, subsequently, a spheroid state. This fusion structure may release or generate single cancer cells with the ability of migration, genomic instability, immune evasion, and increased metastasis[176,182-185]. On the other hand, it is believed that tumor cells must overcome multiple environmental obstacles during metastasis, and metabolic rewiring is widely adapted to achieve successful colonization in distant organs[186]. The adaption of pyruvate, lactate, glutamine, and fatty acids metabolism all contribute a several invasion and migration stages, the circulating stage, and the final colonization step, which represents a potential target for metastasis prevention and treatment[186-189].

Beyond the intrinsic factors of altered signaling and metabolic adaption, extrinsic conditions also participate in the regulation of CRC metastasis. Recently, neutrophil extracellular traps (NETs) formed by decondensed chromatin filaments of neutrophils were implicated in cancer metastasis[174,190]. Clinically, the increased accumulation of neutrophils has been revealed in pre-metastatic organs[191, 192]. Moreover, immunostaining of the CRC tissues demonstrated the existence of NETs in primary and

related metastatic lymph nodes of CRC[193]. Through *in vivo* and *in vitro* assays, NETs were found distributed at the primary site and over the tumor boundary of CRC, whose levels were increased in CRC patients[193-196]. The relevance between NETs and CRC progression and prognosis was illustrated with the assistance of a mouse model and an observational study[197]. Functionally, NETs promote proliferation and the secondary metastasis of CRC, as well as prepare for the adhesion of CTC to the liver or the lung epithelial cells[198-202]. It was also reported that the disruption of NET formation by DNase facilitates the control of CRC metastasis[194,196,199,203,204]. As a pivotal component of the TME, immune cells were found to be involved in CRC metastasis. TAMs have been reported to induce the EMT process for the promotion of the metastatic capability of CRC and CTC-mediated metastasis, which subsequently lead to the production of CCL2 for enhancing macrophage recruitment[205].

APPLICATION OF NANOTECHNOLOGY IN CONQUERING THERAPEUTIC RESISTANCE AND METASTASIS

As mentioned earlier, 2 main reasons result in treatment resistance and metastasis of CRC, namely tumor and environmental factors. Despite the advancements in cancer treatment and the development and design of several drugs or methods to target metastatic sites or reverse drug resistance, only a few such approaches have achieved satisfactory clinical outcomes. The rapid advancements in nanotechnology have provided new methods and options for the treatment of cancer with unique advantages, such as an excellent carrying capacity, better permeability, and the realization of multidisciplinary therapy[206]. In terms of nano delivery methods, compelling reviews have fully illustrated the diversity of nanomaterials currently utilized for cancer therapy, including organic materials, inorganic materials, polymers, and exosomes, *etc*. Exosomes, particularly, have been explored as a potential candidate in therapeutics delivery owing to their intrinsic targeting property, endogenous functionality, and ability to cooperate with a host defense mechanism. So far, exosome-based nanotechnology has achieved marked technological advances and exosome-based nanotheranostic platforms have thus been bloomed [207]. It has been demonstrated that exosome delivery led to higher drug accumulation in target cancer cells and improve molecule stability as well as blood circulation time, thus improving the efficiency of small molecule drugs[208]. Besides, exosomes secreted by different cell types contains a diversity of proteins which facilitates better recognition of targeted cells and reduce nonspecific distribution[209, 210]. In general, nanotechnology-based strategies have been developed to restore drug sensitivity and enhance the anti-tumor efficacy. The detailed advancement in accordance with the mechanism of therapeutic resistance and metastasis, will be discussed in detail in the following section.

Nanotechnology and surgery

Until date, surgery remains the preferred scheme of CRC and the only curative option for malignant bowel obstruction in some cases. As surgical procedures facilitate thorough and timely removal of the primary cancer site, the possibility of disease cure is ensured in most cases. However, 2 major challenges remain, including precise identification of micro-metastatic lesions during the surgery and the generation of a suppressive immune microenvironment after the surgery. Taking advantage of fluorescence imaging technology, researchers have developed novel intraoperative imaging methods that can specifically detect the tumor edge and micro-metastatic sites. For instance, Li *et al*[211] designed a fluorescent probe activated by a CD13/aminopeptidase N (APN), YH-APN, which demonstrated superior tumor-to-normal (T/N) tissue ratios under different conditions. Moreover, owing to the superior ability to distinguish metastatic lesions, even those as small as 1 mm in diameter, image-guided resection, and imaging of metastatic cancer were successfully achieved by spraying YH-APN. Recently, Wang *et al*[212] also used an *in situ* spraying method that enabled precise distinguishing of the tumor edge under the guidance of fluorescent probes. During surgery, the use of real-time fluorescent imaging to detect β-glucuronidase (GLU) activity was achieved by $DCDNO_2$, and the outlines of the tumor were visualized, which guided precise resection of liver cancer.

Regarding the suppressive immune microenvironment, Ji *et al*[20] designed a biopolymer nanogel implant that can be placed in the resection cavity of CRC (Figure 2). Owing to its good tissue adhesion ability and biodegradability, the implant remained in the surgical site and the loaded drugs, including antibody and resiquimod (R848), were released gradually. In the implant group, the residual tumors were eradicated after surgery, which helped inhibit distal tumor metastasis and induced immune-memory effects resisting the re-challenge experiment. Recently, Si *et al*[21] further improved a nanogel implant to an *in-situ*-sprayed gel delivering anti-OX40 antibody, which demonstrated the promising therapeutic potential of CRC. This improved nanogel modulated the suppressive immune microenvironment by inhibiting the activity of cyclo-oxygenase-2 (COX-2); as a result, the loaded aOX40 was gradually released for over 20 days, serving as an immune agonistic antibody. The treated mice displayed resistance to the re-challenge with M38 cells and a remarkable inhibition of the growth of abdominal metastatic tumors[21].

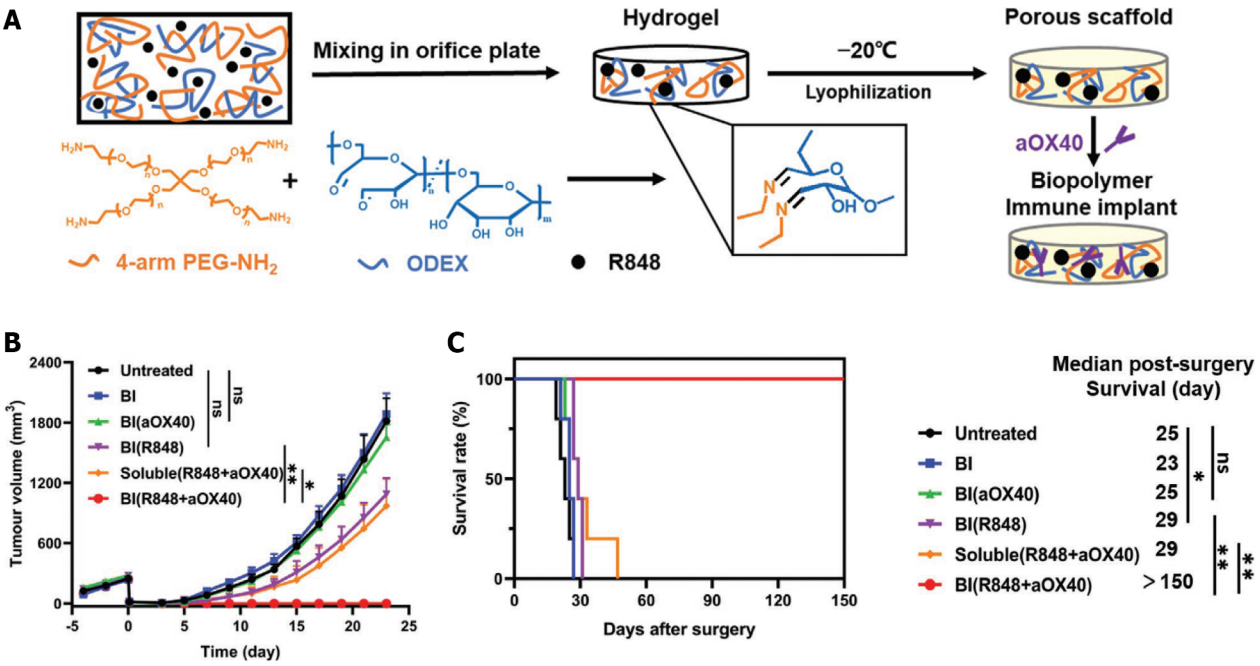

Figure 2 Intraoperative implant of biopolymer exhibited therapeutic effect on colorectal cancer growth. A: Schematic illustration of the implant preparation process; B: Immunoimplant inhibited tumor growth post-surgery; C: Immunoimplant showed survival benefit after tumor re-challenge. Citation: Ji G, Zhang Y, Si X, Yao H, Ma S, Xu Y, Zhao J, Ma C, He C, Tang Z, Fang X, Song W, Chen X. Biopolymer Immune Implants' Sequential Activation of Innate and Adaptive Immunity for Colorectal Cancer Postoperative Immunotherapy. *Adv Mater* 2021; 33: e2004559[20]. Copyright ©The Author(s) 2021. Published by Wiley-VCH GmbH Publications. The authors have obtained the permission for figure using from the Wiley-VCH GmbH Publications (Supplementary material).

Nanotechnology and chemotherapy

The therapeutic strategy of nanomaterials-derived drug delivery system toward overcoming chemotherapy resistance can be generally summarized into 4 aspects: (1) Increasing the drug concentration, accessibility, and permeability; (2) targeting the key genes of drug metabolism; (3) targeting the underlying efflux mechanism of the drug; and (4) the delivery of phytochemicals as an alternative.

The commonly applied strategy for overcoming drug resistance is to increase the local drug concentration. Improved cytotoxicity to human CRC cell lines was achieved through the direct delivery of DOX and 5-FU *via* oleic acid, which reduced the IC_{50} values relative to that with the free-drug forms[206,213]. The codelivery of chemotherapy and targeted therapy by nanomaterials also demonstrated an improved synergistic effect on the CRC cells. Recently, nanoemulsion loaded with paclitaxel (PTX) and BEZ235-a PI3K/AKT/mTOR pathway inhibitor-displayed a greater antitumor effect with remarkably lower IC_{50} when compared with free PTX. The combined therapy with nanoemulsion inhibited the growth of drug-resistant CRC cells either in culture or *in vivo* (Figure 3)[214]. Furthermore, theranostics mediated by magnetic resonance imaging (MRI) was applied to combat drug resistance through precise diagnosis and targeted therapy[215]. The application of the pH-responsive polymer PEALCa nanomicelles loaded with PTX and SPIO avoided the uptake by RES, entered human CRC cells, reached the lysosomes, and released PTX *in vitro*. The drug uptake efficiency could be visualized by MRI, which exhibited a relatively more efficient antitumor effect[216]. Further attempts took advantage of the imaging characteristics of materials to deliver drugs, realize real-time imaging of the drug uptake, as well as to control tumors simultaneously[217-219]. The decreased permeability of a drug is another reason for the development of resistance to it. For instance, Bergers *et al*[186] designed a nanogel loading 5-FU nucleoside floxuridine or other chemical agents, like gemcitabine. Their conjugated nanogel-chemotherapy system exhibited an improved anticancer effect on drug-resistant Caco-2 cells, with enhanced permeability across the gastrointestinal barrier.

Targeting mutations or the altered expression of genes involved in drug metabolism also serves as a promising option to resolve the issue of drug resistance. The upregulated expression of DPD (DPYD) has been implicated in the development of 5-FU resistance, as it catalyzes 5-FU into an inactive metabolite[220,221]. With the intension to reverse the resistance to 5-FU of SW480 cells, Chen *et al*[189] designed hollow mesoporous silica nanoparticles (HMSN) to deliver 5-FU. When grafted with EGF targeting to EGFR-overexpressed SW480/ADR cells, elevated intracellular 5-FU was recorded, which induced apoptosis and cell-cycle arrest, along with the downregulation of DPYD. In addition, restorage of sensitivity to 5-FU was achieved with the use of multi-walled carbon nanotubes and several other nano-based delivery systems with the capability to boost the antitumor efficacy of chemotherapeutics [222,223].

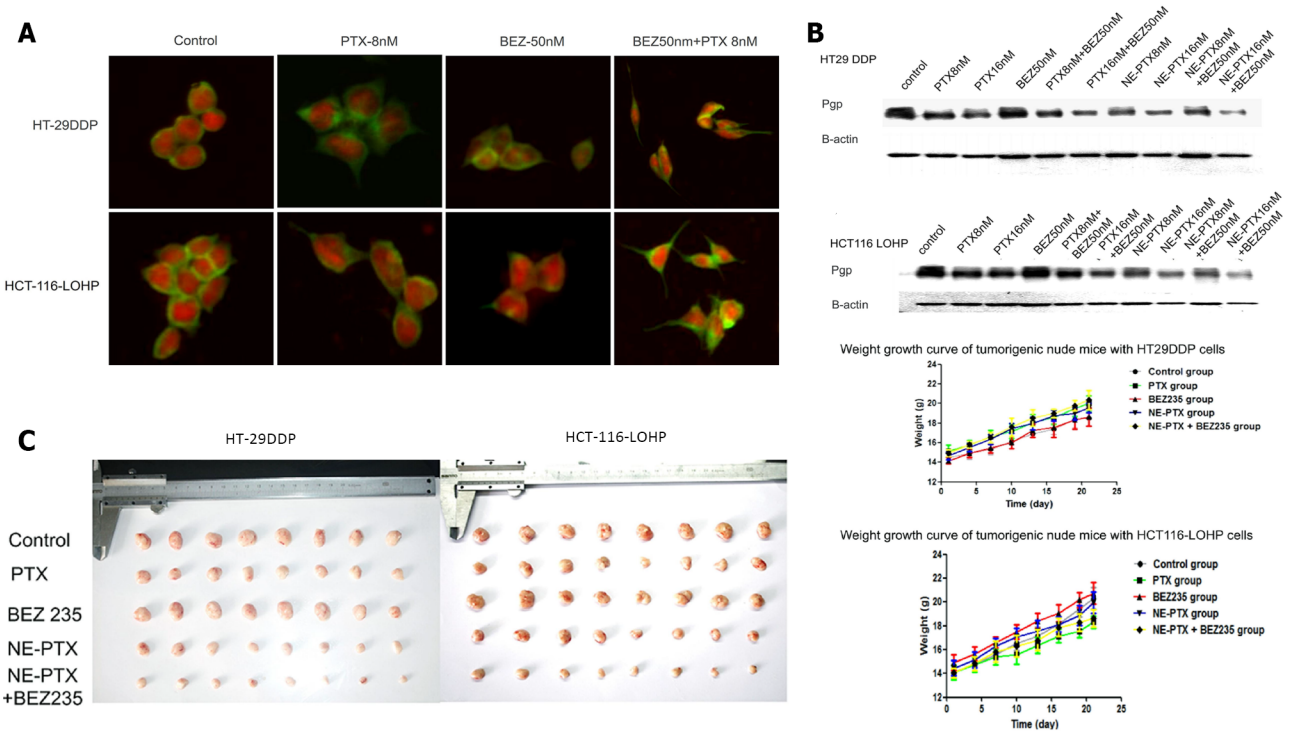

Figure 3 Nanoemulsioned Paclitaxel and BEZ235 reduced multidrug resistance of colorectal cancer. A: Morphological changes of cells treated with nanoemulsioned Paclitaxel and BEZ235; B: Downregulated expression of P-gp after NE-PTX and BEZ235 treatment; C: Synergistic inhibition of CRC growth *in vivo*. Citation: Hu Y, Zhang K, Zhu X, Zheng X, Wang C, Niu X, Jiang T, Ji X, Zhao W, Pang L, Qi Y, Li F, Li L, Xu Z, Gu W, Zou H. Synergistic Inhibition of Drug-Resistant Colon Cancer Growth with PI3K/mTOR Dual Inhibitor BEZ235 and Nano-Emulsioned Paclitaxel *via* Reducing Multidrug Resistance and Promoting Apoptosis. *Int J Nanomedicine* 2021; 16: 2173-2186[214]. Copyright ©The Author(s) 2021. Published by Dove Medical Press Ltd. The authors have obtained the permission for figure using from the Dove Medical Press Ltd. (Supplementary material).

Improvement in the biodistribution and pharmacokinetics of cytotoxic drugs is possible through the adoption of an environmentally responsive nanomaterial-derived delivery system. As reported by Udofot *et al*[224], pH-sensitive liposomal nanoparticles entrapping 5-FU modified with an anti-EGFR antibody (pHLNps-5-FU) exhibited prolonged plasma circulation, while accumulated exposure of pHLNps-5-FU in a tumor site exhibited enhanced anticancer effect relative to that with a free drug[224]. Au nanoparticles (AuNPs) are used to increase the 5-FU efficacy and decrease the related side effects; moreover, AuNPs-based nanoformulation has been reported to enhance the cytotoxicity of 5-FU to CRC cells[225]. Targeting the efflux mechanism to increase the intracellular accumulation of drugs serves as another effective approach to restoring drug sensitivity. For example, NPs designed with a PLGA core and an HA polymer shell loading 5-FU and L3-encoding cDNA (plasmid, pL3) were reported to sensitize CRC cells to 5-FU, as pL3 protein interfered with drug efflux *via* the P-gp regulation[226].

In addition to the abovementioned efforts, alternative therapies have achieved considerable outcomes with the use of naturally available phytochemicals, including curcumin, genistein, lycopene, and resveratrol, among others[227]. Thanks to nanotechnology, novel nanoformulations delivering phytochemicals have realized more efficiency and reduced side effects, which compensate for poor absorption, low solubility, and rapid metabolization of these natural drugs. Liposomes have been applied to enhance the uptake rate of curcumin, and its IC_{50} is lower than that of oxaliplatin, which can reduce the expression of angiogenic factors, increase apoptosis, inhibit angiogenesis, and prevent CRC progression[228]. The simultaneous encapsulation of curcumin and DOX in liposomes have been reported to increase drug accumulation at the CRC sites, exhibiting a higher inhibitory effect compared to that with free DOX[229]. Similarly, the establishment of an amphiphilic block copolymer pluronic/polycaprolactone to encapsulate curcumin demonstrated a better targeting effect on the tumor sites as well as restored the sensitivity of tumor cells to drugs[230]. Considering its abilities of chemosensitization, antioxidation, and anti-inflammation, resveratrol was loaded into gold nanoparticles (GNPs) after tagging with radionuclide technitium ^{99}m; it was rapidly taken up by CRC cells, displaying obvious cytotoxicity to tumor cells and excluding normal colon cells[231,232]. In support, other past studies have confirmed that the combination of resveratrol with 5-FU increases the sensitivity of CRC cells to 5-FU[233-236].

Hui *et al*[237] loaded oxaliplatin into exosomes encapsulating PGM5 antisense RNA 1 by electroporation. The results suggested a significant inhibition of both CRC cell proliferation *in vitro* and tumor growth *in vivo*[237]. Tran *et al*[238] encapsulated aspirin into exosomes derived from colorectal cancer cells and strongly enhanced cytotoxicity of aspirin to cancer cells. Interestingly, this cytotoxic effect was

more pronounced to parental cells of the exosomes, reminiscent of homing effect.

Nanotechnology and radiotherapy

Radiotherapeutic resistance is developed through multiple mechanisms, as illustrated in Section 2. Accordingly, strategies for treating radiotherapy resistance can be categorized into 2 aspects: Investigation of newer biomarkers or signatures to predict radiotherapy sensitivity and the modification of the TME, such as hypoxia. Due to the intrinsic resistance to radiotherapy, it is necessary to establish methods for the detection and identification of sensitive cases before initiating treatment. Recent studies have been able to investigate the genetic signatures and normalize clonogenic assays to predict the radiotherapy response in a more precise and effective manner[239,240]. On the other hand, the modulation of the TME by nanotechnology has demonstrated an improved radiotherapeutic efficacy owing to 3 advantages: An improvement of hypoxia by the loaded cargos, the radiosensitization capacity of some nanomaterials, and the combined application of multiple therapies by using a nanoplatform.

Hypoxia is believed to be one of the most prevailing mechanisms for the development of radioresistance. Methods suggested to restore the tumor oxygen supply before or during radiotherapy include erythrocyte transfusion, erythropoietin administration, and hyperbaric oxygen (HBO) treatment, among others. To date, compelling evidence has demonstrated that hypoxia can be successfully overcome with the use of relevant nanomaterials, as also summarized by several excellent reviews[241-243]. For instance, some simulated oxygen- or water-soluble components can be employed to improve the effect of radiotherapy. In one study, the bioreactive albumin MnO_2 nanoparticles synthesized by nanotechnology produced more oxygen in the hypoxic area, which was helpful to improve the radiotherapy effect[244]. Other strategies adopted so far include the delivery of exogeneous O_2 to the tumor site, *in situ* generations of O_2 and reduced consumption of O_2 in a tumor. Moreover, Tang *et al*[245] developed a nanoeconomizer to overcome radio resistance by regulating the hypoxic environment within tumors. This system functions in two ways: First, it releases NO in the acidic microenvironment to protect endogenous oxygen supply and reduce oxygen consumption; second, the photothermal effect facilitates the clearing of the residual tumor lesions that are resistant to radiotherapy (Figure 4).

Currently, nanoparticles with high atomic numbers, including metal-based NPs, have drawn much interest as radiosensitizers[246-248]. The level of GNPs taken up by CAFs in the TME is higher than that of tumor cells and normal fibroblasts. Therefore, during radiotherapy, GNPs can increase the local radiation dose and reduce the toxic effect on normal tissues, which improves the overall radiotherapy effect. Bromma *et al*[249] compared GNPs uptake by CAFs and DNA damage foci in CAFs to that in normal fibroblasts and HeLa cells and found that GNPs act as an effective tool in cancer radiotherapy to improve the overall efficiency. GNPs are commonly used as a radiosensitizer in *in vitro* experiments, albeit only a few have been used in clinical experiments due to their redox reactivity. Moreover, Guerra *et al*[18] designed an intercomparison of the respective sensitization capacities of GNP and iron oxide nanoparticles in human glioblastoma cells; they found different sensitization mechanisms, suggesting metal-based NPs as effective alternations to eliminate radioresistance.

Finally, through functionalization, the nanomaterials-derived system can be developed to realize disciplinary treatment simultaneously. The combined utilization of radiotherapy and immunotherapy exhibited great synergistic potential in the treatment of cancers. To overcome hypoxia and an immune suppressive microenvironment, Li *et al*[19] constructed a nanoprobe based on quantum dots that emitted in the near-infrared IIb window. By aggregation of nanoprobes in a tumor site, precision radiotherapy was realized with only a few side effects, improved ICD-augmented activation of dendritic cells, and induced robust T cell-mediated antitumor effect. Recently, Guan *et al*[16] engineered a nanoplatform combining radiotherapy and immunotherapy and discovered the potential to remodel the suppressive microenvironment after surgery. They constructed nanomedicine IPI549@HMP-targeted myeloid cells and catalyzed endogenous H_2O_2 to O_2, thereby reversing hypoxia-related radioresistance, which increased susceptibility to anti-PD-L1 therapy and eventually resisted tumor rechallenge.

Wang *et al*[250] developed exosomes from γδ-T cells and found that exosomes from γδ-T cells can specifically target the radioresistant $CD44^+$/high CSCs in nasopharyngeal carcinomas. Moreover, exosomes combined with radiotherapy had a higher therapeutic potency than radiotherapy monotherapy *in vitro* and *in vivo*. Wan et.al found that microRNA-34c-5p (miR-34c) could inhibit malignant behaviors in nasopharyngeal carcinomas. Exosomes overexpressing miR-34c significantly increased radiation-induced apoptosis in nasopharyngeal carcinomas[251].

Nanotechnology and gene therapy

Gene therapy represents a novel tool for conquering therapeutic resistance of CRC in a specific and targeted method, considering that genetic alterations are generally responsible for treatment failure. Strategies involving gene therapy include gene replacement, gene addition, gene expression modifications at the RNA level, and gene editing at the DNA level[252,253]. The combination of molecular biological technologies and nanomaterials has significantly promoted the development of gene therapy for CRC. Pigment epithelium-derived factor (PEDF) protein, a well-accepted tumor suppressor, has been reported to inhibit tumor proliferation and progression through its anti-angiogenesis and pro-apoptotic effects. Recently, Bao *et al*[254] designed a liposome-loading PEDF-DNA decorated with an

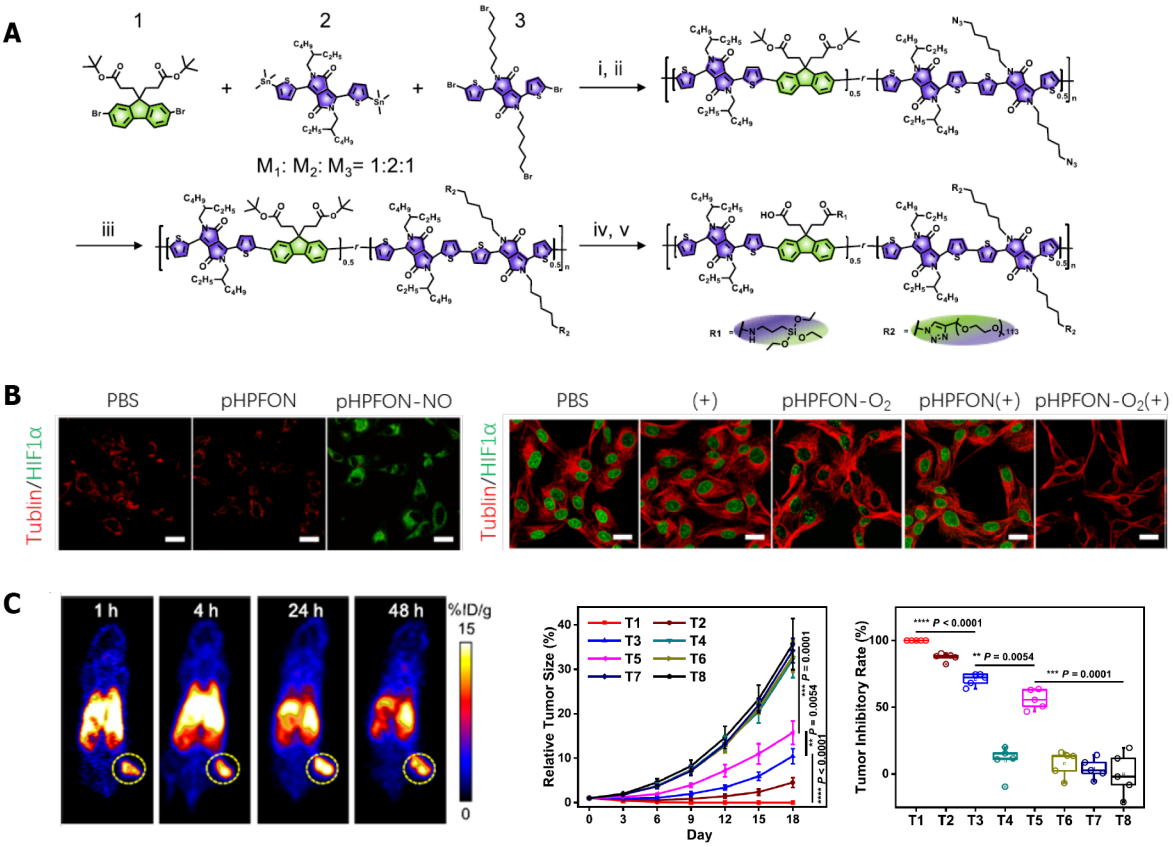

Figure 4 Organosilica-based O_2 nanoeconomizer improved radiotherapy efficacy. A: Design and synthesis of nanoeconomizer; B: pHPFON-NO reduced oxygen consumption (left), and broadened oxygenation (right); C: Multi-modal imaging by pHPFON-NO/O_2, and inhibited tumor growth after pHPFON-NO/O_2 administration. Citation: Tang W, Yang Z, He L, Deng L, Fathi P, Zhu S, Li L, Shen B, Wang Z, Jacobson O, Song J, Zou J, Hu P, Wang M, Mu J, Cheng Y, Ma Y, Tang L, Fan W, Chen X. A hybrid semiconducting organosilica-based O(2) nanoeconomizer for on-demand synergistic photothermally boosted radiotherapy. *Nat Commun* 2021; 12: 523[245]. Copyright ©The Author(s) 2021. Published by Springer Nature Ltd. The authors have obtained the permission for figure using from the Springer Nature Ltd. (Supplementary material).

iRGD peptide and demonstrated the reduced metastatic capability of CRC cells and the induction of apoptosis *in vitro*, as well as the inhibition of lung metastasis and prolonged survival of the animal model.

In addition to delivering DNAs encoding functional proteins, gene silencing therapy is commonly applied in CRC treatment. The methods adopted for gene silencing usually comprise the delivery of antisense oligonucleotide, miRNA, and siRNA, among which siRNA delivery is believed to offer more advantages[255-259]. Moreover, CRISPR/Cas9 technology has achieved a better targeting effect relative to siRNA therapy[260,261]. Recently, Zhao *et al*[262] constructed a cationic liposome-based nanoparticle for purpose of delivering miR-139-5p, decorated with epithelial cell adhesion molecule aptamer on the surface for the targeted treatment of CRC. With the use of cell lines and animal models, the constructed nanoparticle exhibited a satisfactory uptake and targeting ability, while demonstrating a tumor-growth suppressive effect.

On the other hand, siRNA delivery has achieved considerable outcomes through silencing of the key genes related to metastasis and MDR in order to reverse drug resistance of CRC cells[263,264]. To date, several nanocarriers have been developed to deliver siRNAs, which include liposome, PLGA, and RNA nanoparticles. Liposome-derived system is the most common platform for delivering siRNAs, which can protect siRNAs from degradation and kidney clearance while offering the advantages of convenient manufacturing and biodegradability[263]. However, the utilization of liposomes continues to be hindered by issues related to toxicity and release, and PLGA polymers were recently proposed as the preferred agents to realize prolonged release circumventing these obstacles while showcasing better biodegradability and lower toxicity[264-266]. Thanks to rapid development, nucleic acid nanotechnology has gained much attention with its distinct features. Particularly, RNA nanoparticles are widely being used to deliver multiple drugs, thanks to their low toxicity, negative potential, programmable features, and the ability to self-assemble and multi-valent capacity[267]. Rychahou *et al*[268] developed a multifunctional RNA nanoparticle targeting FA and achieved an excellent target effect to CRC metastatic cells mediated by FA-receptor mediated endocytosis, which demonstrated no accumulation in the normal tissues of the targeting organs such as the liver, lymph nodes, and lung. Moreover, the combination of siRNA delivery with RNA nanoparticles was recently applied in the treatment of lung

cancer, breast cancer, and prostate cancer, which effectively inhibited the Survivin expression and slowed the tumor growth *in vivo*[269].

Taking advantage of the abovementioned nanocarriers, siRNA therapy can target various genes or be combined with other treatments. Antigen-presentation cells (APCs) and KRAS are well-known oncogenes responsible for CRC development. Recently, Wan *et al*[270] designed a nanomedicine based on the CRISPR/Cas9 technology, which simultaneously targeted APC and KRAS mutation CRCs. A duplex CRISPR/Cas9 ribonucleoprotein was designed to deliver APC and KRAS sgRNA encapsulated in a phenylboronic dendrimer decorated with hyaluronic acid (HAPD). The resultant nano-complex was administered systematically to Balb/c nude mice, which displayed a synergistic targeting effect on APC and KRAS mutations along with a remarkable reduction in tumor growth. Moreover, the therapeutic capacity for metastasis was further illustrated by the establishment of the orthotopic CRC mouse model and the liver and lung metastasis models, whose survival rates were significantly prolonged (Figure 5)[270]. Intergrin-β1 is one of the key factors involved in CRC metastasis, which is upregulated in CRC cells resistant to regorafenib[271]. The delivery of siRNA-targeting intergrin-β1 to regorafenib resistant CRC cells *via* regorafenib/dimethyldioctadecylammonium bromide (DDAB)-methoxy poly (ethylene glycol) (mPEG)-poly-ε-caprolactone hybrid nanoparticles (HNPs) was found to downregulate the intergrin-β1 expression and induce apoptosis, thereby successfully enhancing the overall sensitivity to regorafenib[271]. P-gp is responsible for drug pump leading to decreased drug concentrations in the cells, which has been proposed as another target of siRNA therapy. The delivery of P-gp siRNA with lipofectamine achieved an efficient control of CRC cells[272]. In addition, the delivery of siRNA-silencing TGF family loaded in tripolyphosphate-decorated chitosan nanoparticles inhibited the formation of liver metastasis of CRCs *in vivo*[273]. The co-delivery of multiple siRNAs or combination with other treatments, such as chemotherapy, can thus be considered as an alternative strategy for the improvement of CRC control[274].

Li *et al*[275] suggested that miR-3940–5p is significantly downregulated in CRC. They loaded miR-3940–5p into exosomes derived from MSCs and transfected them into CRC cells. The results showed significant suppression of EMT and invasion *in vitro*, and inhibition of tumor growth and metastasis *in vivo*. Liu and coworkers evaluated the effect of MSC-derived exosomes transfected with miR-15a on CRC cells. The results indicated the suppression of CRC cell proliferation and tumorigenesis *in vitro* and *in vivo*[276].

Nanotechnology and immuno- and targeted therapies

Currently, it has been well-accepted that nanotechnology offers distinct advantages in improving the efficacy of cancer immunotherapy, which is widely used as the platform that enables the combined utility of immunotherapy with other treatments, along with the reprogramming of the tumor immunosuppressive microenvironment.

Firstly, it has long been established that targeted and immunotherapy can be combined with other anticancer treatments to achieve synergistic effects[6,277-279]. The introduction of nanotechnology to cancer immunotherapy, especially for CRC, is based on the hope of reversing the immunosuppressive microenvironment, inducting a robust immune response and long-term immune memory effect, as well as transforming non-responsive T cells into responsive T cells in the TME for MSS or pMMR CRC[280]. Ni *et al*[281], for instance, constructed a bi-adjuvant nanovaccine (banNV) that delivers the tumor antigen Adpgk, TLR7 agonist R848, and TLR9 agonist ODNs. The combined administration of banNV with anti-PD-1 antibody was found to suppress tumor growth in an orthotopic MC38 mouse model, prolonging the survival rate to 70% on day 48 of treatment. Furthermore, the use of nanomedicine co-delivering photothermal or photodynamic agents with immunotherapy treatment has bene reported to achieve a certain improvement in CRC control. Luo *et al*[282] took advantage of hollow gold nanoshell encapsulating anti-PD-1 peptide into biodegradable poly (D, L-lactic-co-glycolide) nanoparticles (AA@PN), as it overcomes the shortcomings of peptides that have a short half-life, poor stability, and off-target effects. On irradiation by a NIR laser, AA@PN generated photothermal ablation of tumors. In another study, through PD-1 blocking and photothermal ablation *via* NIR laser irradiation, perdurable and enhanced immune response was recorded in the CT26 mouse model that also prolonged the survival rate[282].

He *et al*[283] realized the potential of the combined application of chemotherapy, photodynamic therapy, and ICI therapy through the construction of a nanoscale coordination polymer core-shell nanoparticles-loading oxaliplatin in the core and photosensitizer in the shell. In CRC mouse models, the combined administration of nanoparticles and anti-PD-L1 antibody was found to promote the release of pro-inflammation cytokines and synergistically activate DC and $CD8^+$ T cells, which significantly controlled the local and distant tumor growth, with the primary tumors reducing in size by 2.9% in the PBS-treated group[283]. In addition, nanotechnology offers an optimal platform for radiotherapy and ICI therapy. A nanoscale metal-organic framework was established to enable radiotherapy and checkpoint blockade immunotherapy (indoleamine 2,3-dioxygenase, IDO). The abscopal responses were generated after irradiation, leading to an enhanced immune response in both CRC and breast cancer mouse models[284]. Recently, Guan *et al*[16] developed a novel therapeutic strategy targeting the suppressive microenvironment post-surgery by using the nanomedicine IPI549@HMP, which was composed of a PEGylated $HMnO_2$ (HMP) loaded with PI3-kinase γ (PI3kγ) inhibitor (IPI549). PI3Kγ,

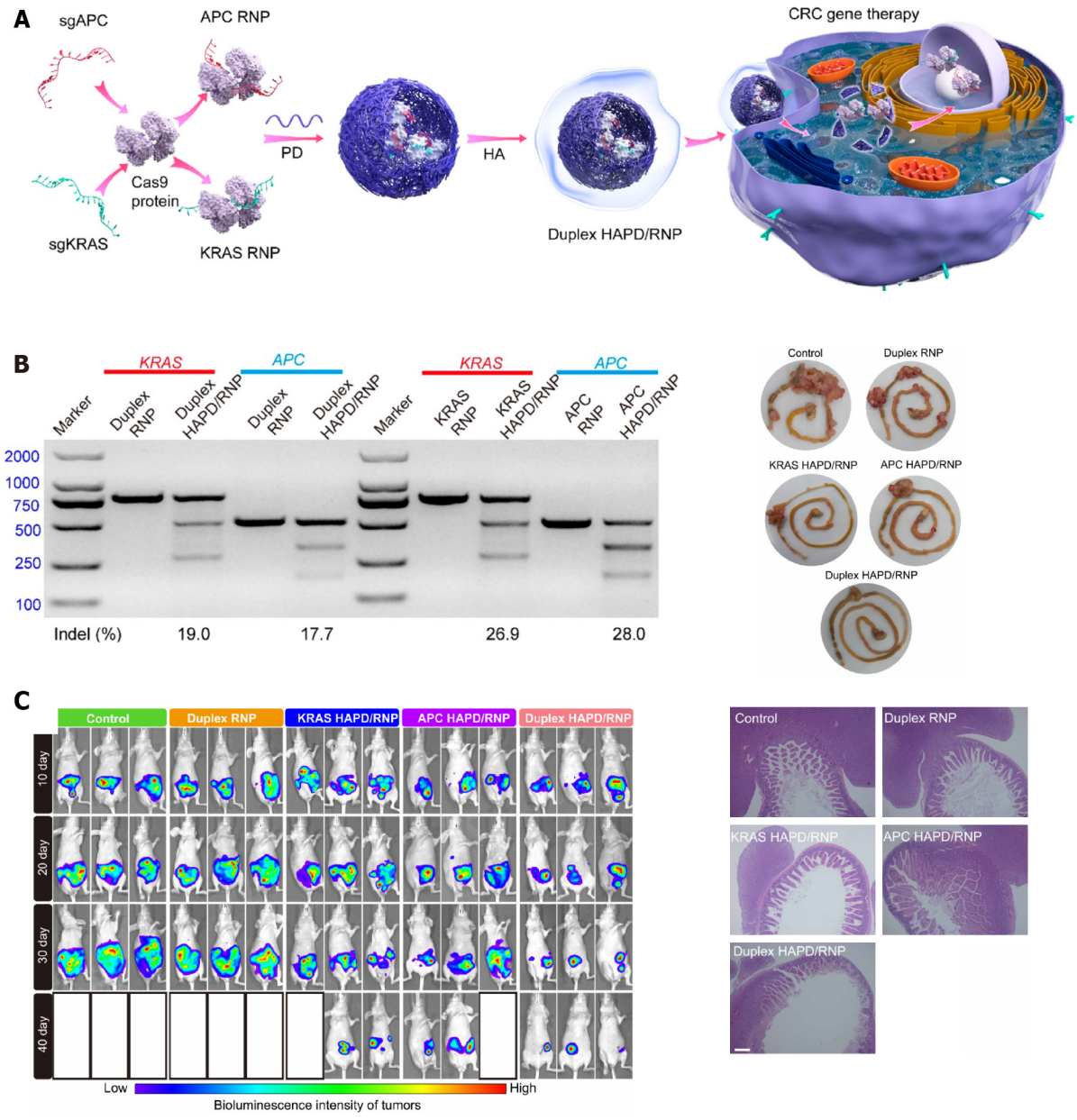

Figure 5 Duplex CRISPR/Cas9 ribonucleoprotein nanomedicine disrupted antigen-presentation cells and KRAS expression to treat colorectal cancer. A: Schematic illustration of preparation of nanoformulations; B: Disruption of antigen-presentation cells and KRAS expression after duplex HAPD/RNP treatment; C: *In vivo* luciferase expression of orthotopic CRC (left), and representative intestine images and H&E staining of intestine sections after nanomedicine treatment. Citation: Wan T, Pan Q, Liu C, Guo J, Li B, Yan X, Cheng Y, Ping Y. A Duplex CRISPR-Cas9 Ribonucleoprotein Nanomedicine for Colorectal Cancer Gene Therapy. *Nano Lett* 2021; 21: 9761-9771[270]. Copyright ©The Author(s) 2021. Reprinted permission from ACS Publications. The authors have obtained the permission for figure using from the ACS Publications (Supplementary material).

which is highly expressed on MDSCs, serves as the target molecule for the specific regulatory effect of IPI549@HMP on myeloid cells. Moreover, this nanomedicine catalyzed H_2O_2 into O_2 by remodeling the hypoxia environment after radiation. In this case, the combined use of IPI549@HMP with anti-PD-L1 antibody exhibited a synergistic effect on the elimination of CT26 cells and the suppression of distant metastasis, which significantly prolonged the survival of IPI549@HMP-treated mice up to 100 d[16] (Figure 6).

On the other hand, nanotechnology also contributes to the modulation of the immunosuppressive microenvironment, as illustrated by several excellent reviews[285-287]. Targeting APCs augments the antitumor effect by inducing a more extensive T-cell response. Zhu *et al*[288] designed self-assembled interwining DNA-RAN nanocapsules (iDR-NCs) that delivered CpG-coding DNA and STAT3-shRNA. The nanocapsules activated APCs by TLR9 signaling and relieved the inhibition of the CpG immunostimulatory effect by silencing STAT3. In an MC38 mouse model, the nanocapsules generated durable T cell responses, demonstrating potent synergistic immunostimulation effect[288]. Importantly, high levels of intratumoral lipopolysaccharide (LPS) are related to a poor response to anti-PD-L1 antibody treatment. Consequently, Song *et al*[289] used polymyxin B (PmB) to trap LPS inside the tumor. In

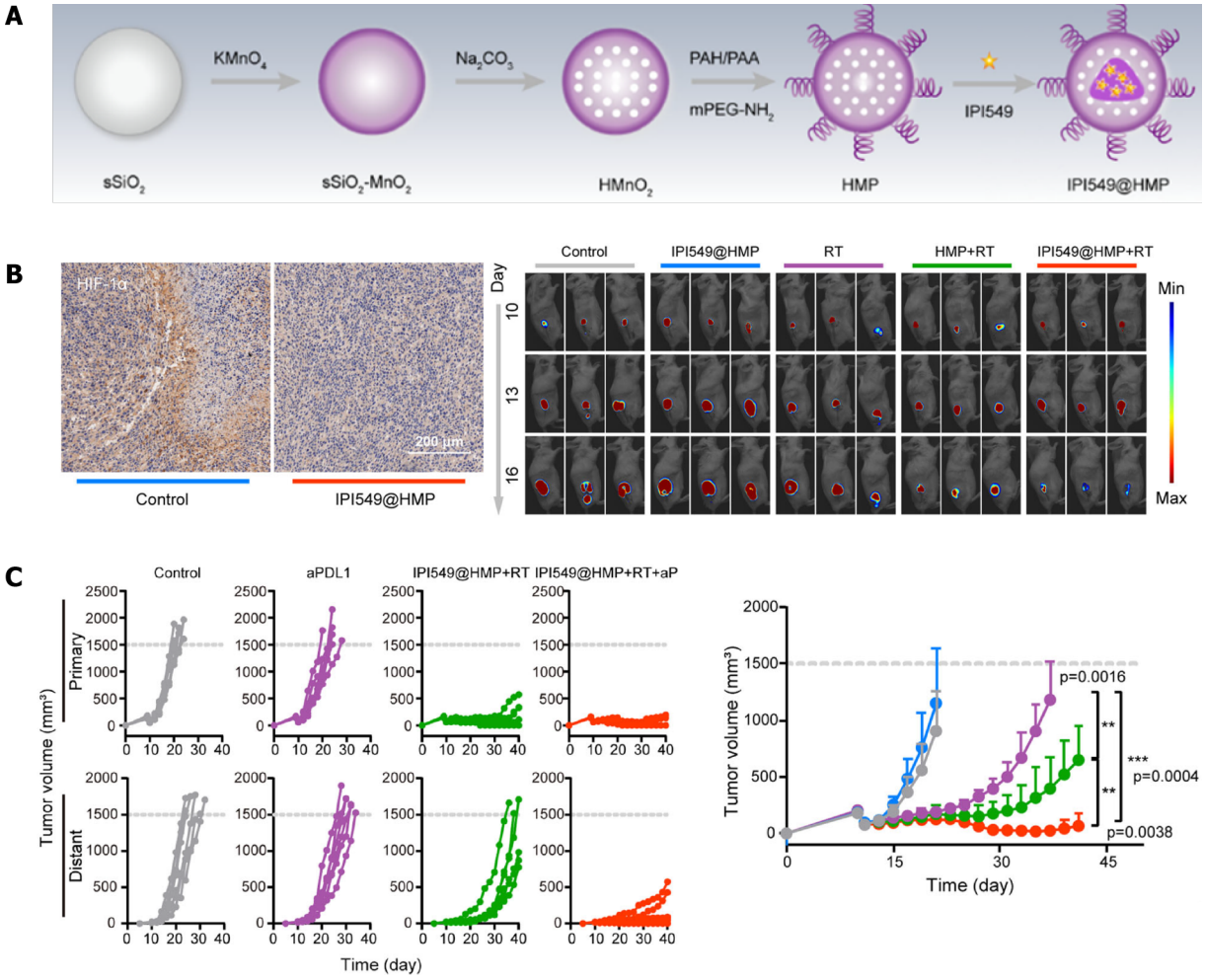

Figure 6 Nanomedicine targeted myeloid-derived suppressive cells exhibited synergistic inhibitory effect on colorectal cancer growth with radiotherapy and PD-L1 blockade. A: Schematic illustration of synthesis of HMP nanoparticles and drug loading; B: IPI549@HMP treatment improved hypoxia environment (left), and augmented radiotherapy efficacy; C: Kaplan-Meier survival curves and tumor growth curves of each groups. Citation: Guan X, Sun L, Shen Y, Jin F, Bo X, Zhu C, Han X, Li X, Chen Y, Xu H, Yue W. Nanoparticle-enhanced radiotherapy synergizes with PD-L1 blockade to limit post-surgical cancer recurrence and metastasis. *Nat Commun* 2022; 13: 2834[16]. Copyright ©The Author(s) 2022. Reprinted permission from Springer Nature Publications. The authors have obtained the permission for figure using from the Springer Nature Publications (Supplementary material).

combination with the TLR4 agonist TAK-242, a remodeled TME was achieved that allowed enhanced infiltration of the T cells in the tumor. The authors designed engineered LPS-targeting fusion protein, the coding sequence of which was loaded into a lipid-protamine-DNA (LPD) system. When combined with an anti-PD-L1 antibody, the nanosystem not only prevented liver metastasis of CT26-FL3 cells but also remarkably inhibited CT26-FL3 tumor cell growth in the liver[289].

In addition to its contribution to the development of immunotherapeutic agents mentioned earlier, nanotechnology serves as a promising tool to enhance the efficacy of targeted therapy. For instance, erlotinib, an orally active inhibitor of EGFR, renders resistance during the treatment of CRC, leading to treatment failure. In addition, Javadi *et al*[290] developed a methoxy polyethylene glycol poly caprolactone loading curcumin and erlotinib. The nanomicelles were administrated in combination and they decreased the expression of αvβ3 integrin while increasing the expression of PDK4 in resistant SW480 cells. Resistance to cetuximab, which is another commonly used anti-EGFR antibody, was found to be reversed with the use of AuNPs (designed as cetuximab-conjugated AuNPs by Hallal *et al*[291]) The application of AuNPs loaded with cetuximab demonstrated an improved endocytosis effect and an enhanced inhibition of the downstream signaling pathway of HT29 cells relative to those with the application of cetuximab alone, suggesting their potential in reversing resistance to EGFR treatment in CRC[291].

Naseri *et al*[292] demonstrated that exosomes derived from CSC-enriched colonospheres (CSCenr-EXOs) significantly increased the IL-12/IL-10 ratio in supernatants of mature DCs. Furthermore, CSCenr-EXOs-loaded DCs effectively promoted T-cell proliferation. Hosseini *et al*[293] loaded exosomes isolated from CT-26 cell line with miR-34a mimic and found that these exosomes could diminish gene expressions related to invasion, angiogenesis and immune evasion and induce cytotoxic T cells *in vivo*.

Nanotechnology-targeted TME

In addition to targeting the immune environment in TME, CAFs should be considered a critical therapeutic target for overcoming the resistance of CRCs. Recently, the functions and regulatory mechanisms of CAFs were reviewed by Kobayashi, who illustrated in detail the clinical implications of using CAFs as biomarkers and targets for controlling gastrointestinal cancer[294]. The authors further recommended 6 strategies to target CAFs: Elimination of CAFs by using CAR T cells or vaccination; reprogramming of cancer-promoting CAFs (pCAFs) into cancer-retarding CAFs (rCAFs); conjugation of antibodies with cytokines; the administration of modified MSCs; blockade of biochemical signaling; ECM-targeting therapy by depleting hyaluronic acid (HA) or the inhibition of lysyl oxidase. As far as CRC is concerned, there has been an increasing interest along with considerable achievement in the application of fibroblast activation protein-targeted vaccination that increases chemotherapy sensitivity, as well as reprograming of CAFs with vitamin D[295,296]. Recently, Ji *et al*[297] designed a novel cleavable amphiphilic peptide (CAP) targeting FAP-α that can load hydrophobic drugs and release the cargo in the presence of FAP-α. The designed drug-delivery strategy, called the CAP-NPs, demonstrated promising specific targeting effects of tumors and achieved therapeutic efficacy for several tumor models[297]. Collectively, much remains to be done to optimally utilize nanotechnology targeting CAFs toward reversing CRC therapeutic resistance and controlling distant metastasis.

CONCLUSION

Resistance to therapies and metastasis are the most critical reasons that ultimately result in treatment failure for CRC patients. The application of nanotechnology in the fields of surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy opens up broad prospects for overcoming drug resistance and controlling CRC metastasis owing to its unique targeted delivery, integration of multiple therapies, and synergistic therapeutic effects. For early-stage CRC, the modulation of the post-surgery microenvironment is worthy of investigation through the simultaneous implantation or spraying with nanomaterials-derived agents so as to improve the overall survival rate of the patients. As resistance to cytotoxic drugs is commonly encountered in a clinical setting, the codelivery of functionalized nanomaterials and natural phytochemicals is a novel alternative to restore the sensitivity of CRC cells. In radiotherapy, it is extremely promising to utilize the instinctive radiosensitivity of nanomaterials to reverse the hypoxic environment and combine it with immunotherapy to improve the overall efficacy of radiotherapy. In delivering gene therapy, nanotechnology offers distinct advantages that may be further studied to realize the specific antitumor effects. With the ongoing research on the immunotherapy mechanism behind CRC, it is necessary to work toward improving the immunomodulation effect of nanotechnology so as to convert non-reactive tumors into reactive tumors. In addition to targeting the immune cells in TME, CAFs can also be considered a promising target by nanotechnology to treat therapeutic resistance. Generally, treatment should not only target tumoral factors including genetic alterations, signal activation, or drug metabolites that lead to drug resistance but also pay attention to modulating the TME so as to eliminate the inhibitory factors and enhance powerful anticancer effects. Meanwhile, it is imperative to maintain a balance between the complexity of composition and structure and in the preparation and fabrication of nanoplatforms. Furthermore, better preclinical models are warranted to reflect the pathogenesis of CRC in order to achieve successful clinical translations of the emerging and established advancements in nanotechnology.

FOOTNOTES

Author contributions: Ren SN and Zhang ZY reviewed the literature and wrote the paper; Zhang ZY designed and draw the figures; Guo RJ, Wang DR, and Chen FF performed the collected data; Fang XD and Chen XB initiated the idea and revised the manuscript; all authors have read and approved the final manuscript.

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MINIREVIEWS

Interferon-lambda: New role in intestinal symptoms of COVID-19

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Abstract

The tremendous public health and economic impact of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a huge challenge globally. There is increasing evidence that SARS-CoV-2 induces intestinal infections. Type III interferon (IFN-λ) has an antiviral role in intestinal infection, with focused, long-lasting, and non-inflammatory characteristics. This review presents a summary of the structure of SARS-CoV-2, including its invasion and immune escape mechanisms. Emphasis was placed on the gastrointestinal impact of SARS-CoV-2, including changes to the intestinal microbiome, activation of immune cells, and inflammatory responses. We also describe the comprehensive functions of IFN-λ in anti-enteric SARS-CoV-2 infection, and discuss the potential application of IFN-λ as a therapeutic agent for COVID-19 with intestinal symptoms.

Key Words: COVID-19; SARS-CoV-2; Interferon-λ; Gastrointestinal infection; Mechanism; Anti-viral response

Core Tip: The tremendous public health and economic impact of the coronavirus disease 2019 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a huge challenge for countries across the whole world. Increased evidences have revealed that SARS-CoV-2 also induced intestinal infection. Type III interferon (IFN), also called IFN-λ, plays an antiviral role in intestinal infection, and has focused, long-lasting, as well as non-inflammatory characteristics. In this review, we summarized the invasion and immune escape mechanisms of the SARS-CoV-2. Furthermore, we concerned about the gastrointestinal impact of the SARS-CoV-2 and the comprehensive role of IFN-λ, also called type III IFN, in anti-enteric SARS-CoV-2 infection.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) was first documented in December 2019, as an outbreak of acute community-acquired atypical pneumonia occurred in Wuhan, China, with unknown etiology. At that time, COVID-19 represented a newly emerged respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which subsequently became a pandemic[1].

Similar to the flu virus, SARS-CoV-2 spreads in small liquid particles from the mouth or nose of an infected person when they cough, sneeze, speak, sing, or breath. These particles range from larger respiratory droplets to smaller aerosols[2]. It is widely reported that coronavirus has the largest non-segmented genome (close to 30 kb) of all RNA virus. This unique genome size enhances the plasticity of CoVs, which alter through mutations and recombination[3]. The first SARS-CoV-2 variant, Alpha B.1.1.7, was reported in the United Kingdom in September 2020[4]. Increasing numbers of variants subsequently appeared, including Beta B.1.351, Gamma P.1, Delta B.1.617.2, and Omicron B.1.1.529[5]. By November 2021, Omicron B.1.1529 had become the current major SARS-CoV-2 variant globally[6]. Because of this strong plasticity in CoV genes, generating high genetic diversity and a high risk of cross transmission, specific treatments remain elusive.

As a systemic disease, SARS-CoV-2 can infect multiple organs, resulting in multiorgan dysfunction in COVID-19 patients. Organs that are infected by SARS-CoV-2 include the lungs, small intestine, gallbladder, kidneys, testes, thyroid, adipose tissue, heart muscle, vagina, breasts, ovaries, and pancreas [7]. The symptoms of COVID-19 include pneumonia, fever, fatigue, intestinal ischemia, and diarrhea[8, 9]. Many studies have reported gastrointestinal (GI) symptoms in COVID-19 patients, including diarrhea, nausea, vomiting, anorexia, abdominal pain, acid reflux, upper GI bleeding, haematochezia, constipation, and melena[10]. Besides, angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2, is highly expressed in intestinal tissue. Moreover, fecal samples from COVID-19 patients have tested positive for SARS-CoV-2 RNA testing. Thus, there is potential for fecal-oral transmission, with SARS-CoV-2 Likely impacting the GI tract[2].

As a part of the innate immune system, type III interferon (IFN-λ) mediates antiviral responses in the epithelial barrier. Moreover, IFN-λ receptor (IFN-λR)1 is preferentially expressed by the epithelial cells of the respiratory, intestinal, and reproductive tracts. The antiviral role of IFN-λ in intestinal infection has focused, long-lasting, and non-inflammatory characteristics. Consequently, many studies have explored the potential of applying IFN-λ in COVID-19[11]. Continuing research on SARS-CoV-2 showed that it is also expressed in the intestinal tract. In particular, IFN-λ might have crucial roles in SARS-CoV-2 intestinal infection.

In this review, we summarize the invasion and escape mechanisms of SARS-CoV-2, and both the direct and indirect influence of SARS-CoV-2 on intestinal homeostasis. We also consider how IFN-λ impacts intestinal infections of SARS-CoV-2, and focus on the new functions of IFN-λ in COVID-19 with intestinal symptoms.

SARS-CoV-2

Coronaviruses belong to the subfamily coronavirinae of the coronaviridae family, and have caused three pandemic outbreaks globally[12]. The subfamily coronavirinae consists of four genera; α, β, γ, and δ-coronaviruses. α and β-coronaviruses usually cause respiratory illness in humans and gastroenteritis in animals. In comparison, γ and δ-coronaviruses mostly infect birds, and, sometimes, mammals. According to genome sequence analysis, SARS-CoV-2, which was the third virus to create a pandemic, is classified in the β-coronavirus genome[12].

The four structural proteins of SARS-CoV-2 are essential for infections to occur. They consist of the spike (S) surface glycoprotein, membrane (M) protein, envelope (E) protein, and nucleocapsid (N) protein. The S protein is cleaved by host proteases, and allows SARS-CoV-2 to attach to host cells[13]. Both E and M proteins induce S protein to reside in the Golgi/ER Golgi intermediate compartment (ERGIC) compartment, and subsequently, together, regulate N-glycosylation maturation of the S protein [14]. N structural proteins are responsible for viral gene binding, compression, and packaging. In addition, they contribute to RNA transcription, and achieve the innate immune escape of the host through the N protein directly targeting and sequestering G3BP1 and host mRNA[15]. Once infected, SARS-CoV-2 mainly induces T cell responses; however, the generation of high-titer neutralizing antibodies by B-cell expansion and maturation might be limited[16,17]. To explore potential treatment

directions of SARS-CoV-2, it is important to learn its route of infection and mechanisms of immune evasion.

Infection mechanism of SARS-CoV-2

Zhou *et al*[18] first confirmed that SARS-CoV-2 only infects HeLa cells that express ACE2, which is used as a SARS-CoV-2 receptor in patients[18]. Although there are two independent ways to mediate the infection of SARS-CoV[19,20], the entry of SARS-CoV-2 involves ACE2-receptor-mediated transmembrane serine protease 2 (TMPRSS2)-dependent membrane fusion[21] (Figure 1).

SARS-CoV-2 is an enveloped virus containing a large N encapsidated positive sense RNA genome. S trimers with closed and open prefusion structures usually protrude from the lipid bilayer of SARS-CoV-2. They are distributed randomly on the viral surface to bind to the receptor, ACE2, and, subsequently, mediate viral uptake and fusion. The receptor binding domain (RBD) site is occluded by three copies of the N-terminal domain when in the closed prefusion. In the open prefusion, one or multiple RBDs lift up to expose the receptor binding site[22]. There are two main subunits, S1 and S2. S1 binds ACE2, while S2 triggers membrane fusion of the virus to the cell. In a typical SARS-CoV-2 infection process, S trimers must be activated by cellular protease-mediated cleavage at two distinct sites, S1/S2 and S2'. S2' is responsible for triggering virus-cell membrane fusion. TMPRSS2 is expressed on the cell surface, and activates S trimers, which promote the virus to enter the plasma membrane in a pH-independent way [23]. Finally, the encapsidated genome buds into the ERGIC to form virions, which are trafficked to the plasma membrane and released[22] (Figure 1).

Evasion mechanism of SARS-CoV-2

The first immune evasion of SARS-CoV-2 is related to the delayed occurrence of IFN-I[24]. As the first line of host defense against virus infections, IFN-I response is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) *via* host pattern recognition receptors (PRRs). SARS-CoV-2 RNA is detected by various cytosolic sensors, including retinotic acid-inducible gene 1 (RIG-I/DExD/H-box helicase 58) and melanoma differentiation-associated gene 5 (IFN induced with helicase C domain 1). These sensors induce an antiviral signaling cascade to occur[25]. A subset of IFN-stimulated genes (ISG) is produced by the IFN-I response. These ISGs, along with other downstream molecules controlled by IFN-I, directly inhibit virus replication and recruitment, and activate diverse immune cells[26]. However, viral proteins (NSP3/papain-like protease) encoded by the SARS-CoV-2 genome selectively cleave interferon regulatory factors (IRF) 3 protein directly during SARS-CoV-2 infection, resulting in a blunted Type-I IFN response[27]. M proteins on SARS-CoV-2 also target mitochondrial antiviral signaling proteins (MAVS), and impair the aggregation and recruitment of downstream signaling components[28]. In parallel, stress granule analogues, formed by the N protein, competitively bind G3BP1 to inhibit the innate immune response[15]. Furthermore, SARS-CoV-2 nsp13 (helicase), nsp14 (exonuclease), nsp15 (endoribonuclease), and accessory protein orf6 block IFN-I production by antagonizing upstream signaling pathways[29,30].

The second method of evasion is the upregulation of human inhibitory receptors, which modulate natural killer (NK) cell-mediated cytotoxicity. This process causes the exhaustion of NK cells, reducing their ability to clear viral infection[31].

Factors contributed to the severity of SARS-CoV-2

A recent meta-analysis of COVID-19 patients showed that physical activity could reduce the risk of COVID-19 infection. Physical activity strengthens the immune system, and allows cardiopulmonary and musculoskeletal adaptations[32]. Moreover, adherence to a high-quality healthy diet has been linked to a reduced risk of COVID-19 infection and lower hospitalization rates. Mediterranean-style dietary patterns are associated with a lower risk of death from respiratory infections, including COVID-19, while plant-based diets have been associated with lower COVID-19 infection rates[33]. Age is also an important factor in severe COVID-19 illness and its adverse consequences. Statistically, the case fatality ratio for COVID-19 increases with age[34]. There has also been widespread concern about whether pregnant women are more susceptible to SARS-CoV-2. However, to date, there are no data supporting an increased susceptibility to SARS-CoV-2 due to pregnancy. Nevertheless, the risk of death in pregnant women with COVID-19 is significantly increased[35]. There is also a nonlinear association between ambient temperature and the risk of infection with SARS-CoV-2. Specifically, the risk of infection with SARS-CoV-2 increases significantly at temperatures between 0 and 10 °C[36].

INTESTINAL INFECTION OF SARS-CoV-2

As a receptor of SARS-CoV-2, ACE2 is expressed in most organs. Consequently, COVID-19 is capable of infecting multiple organs, in addition to the respiratory tract[37]. It has been confirmed that ACE2 is abundantly expressed in GI glandular epithelial cells[38]. There is also clinical evidence that the intestine is another target organ. When SARS-CoV-2 infects the GI tract, it disrupts gut microbiota and induces other GI symptoms. Current research on SARS-CoV-2 invasion of the gut is focused on (1)

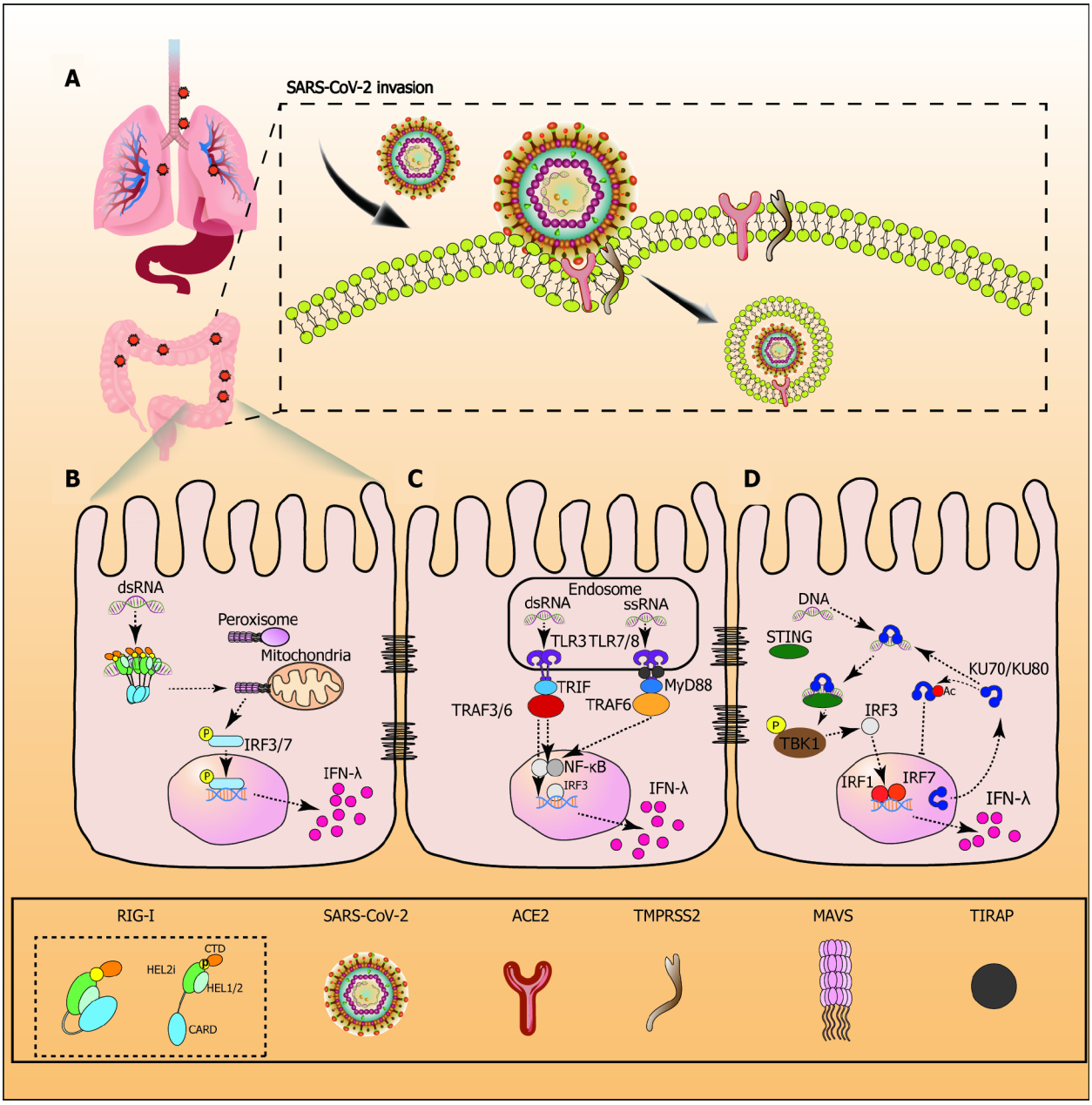

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Figure 1 Infection mechanism of severe acute respiratory syndrome coronavirus-2 and retinotic acid-inducible gene 1. A: Process of severe acute respiratory syndrome coronavirus-2 invasion in the gastrointestinal tract; B: Retinotic acid-inducible gene 1 (RIG-I) contains a pair of caspase activation and recruitment domains (CARDs), an ATPase motor domain (Helicase domain) that consists of two RecA domains (Hel1 and Hel2), and an alpha-helical insertion domain (Hel2i) connected *via* a Pincer motif (P) and a C-terminal domain. When RIG-I is in the inactivated state it clasps the CARDs against the surface of Hel2i. Once engaged with viral RNA, these pathogen RNAs trigger conformational changes that anchor the RNA tightly inside the RIG-I receptor and trigger the release of the CARDs[91]. Activated RIG-I interacts with mitochondrial antiviral signaling proteins to recruit phosphorylated interferon regulatory factor (IRF)3/7 in the nucleus, subsequently inducing interferon-λ (IFN-λ) production; C: Toll-like receptor (TLR) 3 interacts with dsRNA at opposite ends of the horseshoe ring in the endosome. TLR3 recruited TIR-domain-containing adapter-inducing interferon-β (TRIF) *via* the myeloid differentiation primary response 88 (MyD88) independent pathway. TRIF then recruits and activates the tumour necrosis factor receptor associated factor (TRAF) 3/6, inducing the recruitment of IRF3 and nuclear factor kB (NF-κB) in the nucleus. After TLR7/8 recognizes viral ssRNA in the endosome, the receptor-proximal membrane protein TIRAP detects the dimerized TIR domain of TLR7/8, and stimulates MyD88 to interact with it. MyD88 subsequently recruits TRAF6, leading to the recruitment of IRF3 and NF-κB in the nucleus. Once IRF3 binds to the promoter, IFN-λ gene expression is initiated[92,93]; D: Ku80 is co-localized with Ku70. Ku70 translocates from the nucleus to the cytoplasm, and recognizes cytosolic viral DNA. Subsequently, Ku70 with the DNA interacts with stimulator of interferon genes to phosphorylate TANK-binding kinase 1. Finally, IRF3 is activated, and produces the strong expression of IRF1and IRF7, which induce IFN-λ expression. Acetylation increases Ku70 accumulation in the cytoplasm and promotes DNA-mediated Ku70-dependent IFN-λ1 induction[94]. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IRF3/7: Interferon regulatory factor 3/7; IFN-λ: Interferon-λ; TRAF: Tumour necrosis factor receptor associated factor; TLR: Toll-like receptor; TRIF: TIR-domain-containing adapter-inducing interferon-β; NF-kB: Nuclear factor kB; TBK1: TANK-binding kinase 1; STING: Stimulator of interferon genes; MyD88: Myeloid differentiation primary response 88.

changes to the gut microbiome; and (2) whether GI symptoms are caused by direct infection of SARS-CoV-2 or the consequence of a systemic immune activation.

Impact of SARS-CoV-2 on the gut microbiome

The gut microbiome consists of bacteria, fungi, viruses, and other microorganisms that dynamically interact with environmental factors to shape the intestinal mucosal immune system[39]. The intestinal bacteria of healthy individuals are mainly composed of Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes. Intestinal microbiota is highly resilient to external disturbances, enabling the host to retain key species for a long time and maintain intestinal homeostasis. However, severe external influences cause microbial ecosystems to move from a stable state to an unhealthy stable state associated with disease[40]. A comparison of gut microbiota in COVID-19 patients and healthy people showed that the relative abundance of gut bacterial groups was higher in COVID-19 patients (including *Ruminococcus gnavus*, *Clostridium ramosum*, *Coprobacillus*, *Akkermansia muciniphila*, and *Eggerthella lenta*). Furthermore, the abundance of *Alistipes shahii* was comparatively lower in COVID-19 patients, along with several butyrate producers (including *Roseburia intestinalis*, *Eubacterium hallii*, *Ruminococcus bromii*, and *Faecalibacterium prausnitzii*)[41]. Gut microorganisms regulate mucosal sites in the distal part of the intestine *via* metabolites. These metabolites access other organs through the bloodstream, to induce immunomodulatory, immunoglobulin, and anti-inflammatory effects[42]. One cohort study showed that the lower abundance of *B. adolescentis*, *F. prausnitzii*, *E. rectale*, *R. (Blautia) obeum* and *D. formicigenerans* in the COVID-19 cohort might be associated with the decreased secretion of anti-inflammatory cytokines or increased secretion of inflammatory cytokines[43]. Another clinical study found that GI infection with SARS-CoV-2 differed to that without infection. Significant dysbiosis of the microbiome was documented in COVID-19 patients, with high levels of pathogenic bacteria[44]. In general, the intestinal microbiota of active GI infections with SARS-CoV-2 were mainly characterized by the enrichment of opportunistic pathogens and reduction of beneficial bacteria[45]. One study of COVID-19 patients showed that changes to the gut microbiome are correlated with the severity of COVID-19. In particular, the abundance of *Coprobacillus*, *Clostridium ramosum*, *and Clostridium hathewayi* is correlated with the severity of COVID-19. Furthermore, *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacterodies massiliensis*, and *Bacteroides ovatus* downregulate the expression of ACE2 in the murine gut, correlating with SARS-CoV-2 load in fecal samples[46].

In addition to SARS-CoV-2 directly infecting the GI tract, it affects the functioning of the GI tract through other organs. For instance, Dhar *et al*[47] showed that gut microbiota affected pulmonary health *via* vital cross-talk between the gut microbiome and lungs which is called the "gut-lung axis." However, the gut-lung axis is bidirectional; consequently, when SARS-CoV-2 infects the lungs, the gut microbiome might also be affected, including disruption[47].

Systemic immune activation

When infected by SARS-CoV-2, activated CD8+ IEL migrate to the intestinal mucosa[48]. The infected cells block IFN signaling, and exhibit strong pro-inflammatory responses by strongly activating the nuclear factor kB (NF-κB)/tumour necrosis factor pathway. In parallel, bystander cells not infected by SARS-CoV-2 mount an IFN-mediated response[49]. Although human plasmacytoid predendritic cells (pDCs) are not infected by SARS-CoV-2, pDCs may be activated by SARS-CoV-2 through IRAK4 and UNC93B1-dependent pathways, inducing high levels of type I and type III IFNS[50].

In healthy cells, CCL5, CXCL1, CXCL10, and CXCL11 recruit immune cells and pro-inflammatory chemokines. However, these four chemokines were significantly upregulated in SARS-CoV-2-infected epithelial cells in a trial of biomimetic human gut-on-chip system[51]. Interestingly, in a clinical cohort study, a subset of COVID-19 patients with GI symptoms had small intestinal epithelial cells infected with SARS-CoV-2. Compared to patients without GI symptoms, key inflammatory genes [including IFN-γ, CXCL8, CXCL2, and interleukin (IL)-1B] were downregulated in these cells, and the frequency of pro-inflammatory dendritic cells was reduced in immune subsets[52]. The authors also recorded lower mortality among COVID-19 patients with GI symptoms. Thus, a stronger immune response was induced in SARS-CoV-2 infected human intestinal tissue, reducing the efficiency of viral replication[53]. A recent study of rhesus monkeys infected with SARS-CoV-2 proposed that antiviral immunity was activated and the inflammatory response was enhanced at the early stage of intestinal infection. This response damages the intestinal barrier; however, the intestine was repaired and inflammatory response decreased at the later stage, leading to mild intestinal pathological damage[54]. Intestinal IFN-related antiviral response and neutrophil related pathways are activated at the early stage. This process damages Paneth cells and the intestinal barrier, enhancing the inflammatory response. Nevertheless, the increasing number of Paneth cells and B cells that inhibit neutrophil-chemotactic cytokines CXCL8 and IL-1B at the later stage, repair the homeostasis of the intestinal barrier, inhibiting inflammation and tissue damage[54].

TYPE III IFN

IFNs interfere with viral replication and induce inflammation, and are essential for mobilizing immune responses to SARS-CoV-2 infection[55]. There are three types of IFNs, and each has its own distinctive receptors. Type I IFN consists of at least 32 functional subtypes, including IFN-α subtypes (13 in humans and 14 in mice), IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ξ[56]. The type I IFN receptor (IFNR) is mainly expressed in immune cells. IFN-α/β responses to viral infections result in immunopathology. However, type I IFN also induces the infiltration of inflammation[57]. The mechanism of SARS-CoV-2 innate immune evasion depends on dysregulated IFN-I production, which contributes to robust early SARS-CoV-2 replication[31]. Type II IFN (IFN-γ) is a proinflammatory cytokine[58-60]. IFN-λ consists of four subtypes in humans (λ1, λ2, λ3, λ4), and its receptor is localized to epithelial cells and a subset of immune cells, including neutrophils. IFN-λ is widely believed to control pathologic microbes in the intestinal epithelium, and promotes the healing of the colonic epithelium without inducing an excessively strong inflammatory response[61].

Immune responses triggered by PAMPs are key factors for pathogen defense. Differences in the location of PAMP recognized by some PRRs affect the type of IFN produced. PRRs in cytosol include RIG-I like receptors (RLRs), cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), and Ku70, whereas PPRs in the endosome are toll-like receptors (TLRs)[62]. cGAS mainly induces the expression of pro-inflammatory cytokines and the IFN-I gene through the stimulator of interferon genes (STING)-TANK-binding kinase 1 (TBK1)-IRF3/NF-κB signaling pathway. In comparison, RLRs, TLRs, and Ku70 induce the IFN-λ gene[63]. The downstream signaling pathways of IFN-λ is similar to IFN-I, which generates an antiviral response through the JAK-signal transducer and activator of transcription (STAT) pathway. In mitochondria or peroxisome, RLRs interacts with the MAVS, resulting IRF 3 and 7 being recruited into the nucleus. IRF3/7 binds to the promotor of IFN-λ to initiate the gene expression of the IFN-λ family. Activated TLR7, 8 and 3 signal is transduced by Myeloid differentiation primary response 88 and TIR-domain-containing adapter-inducing interferon-β. This process results in the recruitment of IRFs and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) in the nucleus. Subsequently, the expression of IFN-λ gene is enhanced[64]. The cytoplasmic translocation of Ku70 is the first step in initiating IFN-λ1 secretion, which facilitates the interaction between STING and Ku70. Activated STING promotes TBK1 phosphorylation, which activates IRF3. Since IRF3 is activated first, it produces the strong expression of IRF1 and IRF7, which are not endogenously expressed in cells. Subsequently IFN-λ1 is induced in the nucleus[65] (Figure 1).

In contrast to the type I IFNR, the IFN-λR is preferentially expressed by epithelial cells and neutrophils. The IFN-λR consists of IFN-λR1 (also called IL-28Rα) and IL-10R2. IFN-λR1 specifically binds IFN-λ, whereas IL-10R2 binds with cytokines in the IL-10 family. When IFN-λ signaling is activated by IFN-λ, the receptor-associated kinases JAK1 and TYK2 phosphorylate STAT1 and STAT2. IFN-stimulated gene factor 3 (ISGF3) is activated by IFN-λ, and is a trimeric complex that is formed by phosphorylated STAT1, STAT2, and IRF9. ISGF3 binds IFN-stimulated response elements in the nucleus to promote the transcription of hundreds of antiviral effector ISGs[66]. In addition to the STAT1-dependent pathway, IFN-λ promotes tissue healing by the upregulation of collagens *via* the MAPK-dependent pathway[67] (Figure 2).

IMPACT OF THE TYPE III IFN ON SARS-CoV-2 INFECTION OF THE GUT

Diarrhea is the most common GI symptom in COVID-19 patients. It is associated with changes to gut microbiota, malabsorption, and inflammation. It is also associated with the release of virulent proteins and toxins, and viral-induced intestinal fluid and electrolyte secretion. Of note, calprotectin, an inflammatory hallmark, was secreted by infiltrated neutrophils in COVID-19 patients with diarrhea[10].

The rapid initiation of the innate immune response after pathogen encounter is the result of infection and survival of the host. Highly expressed IFNs induce the secretion of ISGs, which is a hallmark of the innate immune response. ISGs induce various cell-intrinsic antiviral responses, such as blocking the translation and induction of apoptosis, while recruiting of immune cells and stimulating effector functions[68]. Compared to IFN-I, the effects of IFN-λ are delayed but last longer. IFN-λ is transcribed and translated at higher rates in intestinal organoids. Furthermore, IFN-λ is preferentially induced on the mucosal surfaces of both the intestine and lung[69]. Furthermore, cells cultivated under polarizing conditions exhibit high IFN-λ responsiveness[70]. Pretreatment with IFN-λ in Vero E6 cells showed dose-dependent inhibition of SARS-CoV-2[71]. IFN-λ promotes epithelial healing in patients infected with SARS-CoV-2 with acute colonic inflammation and tissue damage[72]. Moreover, IFN-λ treatment directly improves the proliferation and regeneration ability of differentiated GI epithelial cells through Lgr5+ intestinal stem cells (ISC), leading to the recovery of intestinal barrier integrity[73]. Furthermore, IFN-λ protects epithelial cells from enteric viral infections, indicating that IFN-λ could be used to treat SARS-CoV-2 infection of the GI tract.

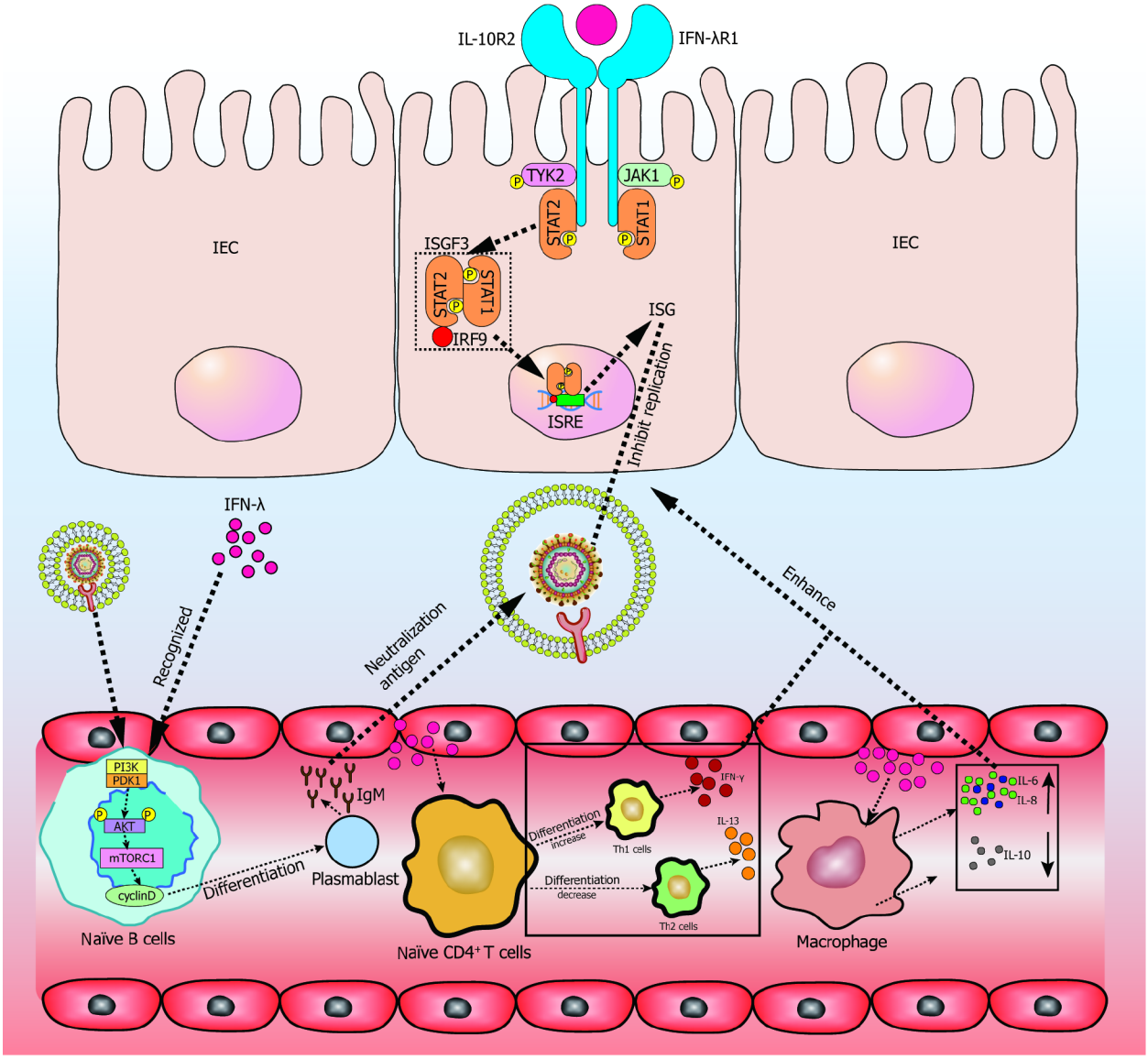

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Figure 2 When interferon-λ is recognized in intestinal epithelial cells, JAK1 and TYK2 phosphorylate and activate phosphorylated signal transducer and activator of transcription 1 and signal transducer and activator of transcription 2. Interferon (IFN)-stimulated gene factor 3 is formed by phosphorylated signal transducer and activator of transcription 1, signal transducer and activator of transcription 2, and interferon regulatory factor 9. It then binds to IFN-stimulated response elements in the nucleus to promote the transcription of IFN-stimulated genes (ISGs). ISGs inhibit the RNA replication of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Recognition of IFN-λ in naïve T cells promotes the differentiation of Th1 cells while inhibiting Th2 cells, resulting in increased IFN-γ and lower interleukin (IL)-13. In peripheral mononuclear blood cells (PMBCs), IFN-λ induces higher IL-6 and IL-8, while reducing IL-10. The effects of IFN-λ on naïve T cells and PMBCs enhance the antiviral effect of ISGs in intestinal epithelial cells. IFN-λ and pathogens are co-recognized by naïve B cells, and promote the differentiation of naïve B cells to plasmablasts through the phosphoinositide 3-kinase-phosphoinositide dependent kinase-1 pathway. This action produces large amounts of immunoglobulin M (IgM). IgM acts as a neutralizing antibody in the intestinal tract of SARS-CoV-2 infection. STAT1: Signal transducer and activator of transcription 1; STAT2: Signal transducer and activator of transcription 2; IL: Interleukin; IFN-λ: Interferon-λ; IEC: Intestinal epithelial cell; ISGF3: IFN-stimulated gene factor 3; IRF9: Interferon regulatory factor 9; ISRE: IFN-stimulated response element; ISG: IFN-stimulated genes; PI3K: Phosphoinositide 3-kinase; PDK1: Phosphoinositide dependent kinase-1; mTORC1: Mechanistic target of rapamycin complex 1; IgM: Immunoglobulin M.

IFN-λ-mediated anti-SARS-CoV-2 response

IFN has been confirmed to be the first line of innate immunity, producing an antiviral response when the virus infects the intestinal tract. SARS-CoV-2 infects, replicates, and implements de novo infectious virus production in human intestinal epithelial cells (IECs). Furthermore, IFN-λ has huge potential for treating SARS-CoV-2. One study showed that the JAK-STAT1 dependent pathway of IFN-λ is more critical for anti-SARS-CoV-2 activity than IFN-I. Pretreatment IECs with IFN-λ resulted in a substantial decrease in the number of SARS-CoV-2 infected cells and viral replication[74]. Type I and II IFN upregulate antiviral ISGs when the virus infects cells, with ACE2 mRNA expression simultaneously increasing. Nevertheless, IFN-λ upregulates antiviral ISGs, whereas ACE2 mRNA is only marginally elevated[75]. Furthermore, one of the mechanisms by which IFNs restrict viral replication is to inhibit the translation process, which inhibits the expression of structural proteins, with SARS-CoV-2 being

more sensitive to IFN-λ. Compared to type I IFN, IFN-λ maintained a more consistent anti-SARS-CoV-2 infection state, with viral replication being inhibited for more than 72 h after drug withdrawal[76]. As for exogenous IFN-λ, pretreated IFN-λ significantly reduces the SARS-CoV-2 burden in the air-liquid interface of the lung model. The therapeutic administration of IFN-λ is also effective at restricting SARS-CoV-2 production in cultured human lung cells[77,78]. Another vitro experiment showed that recombinant bovine IFN-λ produced in HEK-293 cells effectively prevents SARS-CoV-2 infection toward VERO cells[79].

Effect of IFN-λ mediated recruitment and activation on immune cells

It remains unclear whether IFN-λ directly or indirectly regulates the immune response after SARS-CoV-2 infection. To date, IFN-λ1-4 is considered to regulate the immune response, including the upregulation of IL-6, IL-8, and the downregulation of IL-10 in peripheral blood mononuclear cells (PBMCs)[79]. It also alters Th1/Th2 T-cell balance, reduces IL-13 production by T cells[80], and induces ISGs in B cells and plasmacytoid dendritic cells, as well as in CD8 T cells, especially effector memory cells (TEMRA)[81] (Figure 2). As an anti-inflammatory factor, IL-10 induction in macrophages provides a powerful pathogen immune escape mechanism, which causes chronic infection[82]. IFN-λ stimulates a stronger innate immune response by upregulating IL-6 and IL-8, and by reducing IL-10 secretion in PBMCs, which promote an antiviral response[79]. *In vivo*, IFN-λ promotes the differentiation of initial T cells to Th1 cells, rather than Th2 cells, thus activating a stronger immune response, which combats the invasion of the gut by pathogens[80]. Furthermore, in the intestinal tract (unlike IFN-I), IFN-λ exhibits a compartmentalized response to virus infections because of the restricted expression of IFN-λR, which is preferentially expressed on IECs. IFN-λR expression has also been reported in NK cells, T cells, B cells, and pDCs[83]. Interestingly, IFN-λ induces the mechanistic target of rapamycin complex 1 pathway, leading to the differentiation of naïve B cells in plasmablasts by activating phosphoinositide 3-kinase *via* JAKs in an IRS-dependent, but STAT-independent, manner[84]. Thus, IFN-λ likely promotes the release of antibodies and increases the capability of antiviruses. These immune cells activate strong immune responses in the body to resist pathogen invasion and maintain intestinal homeostasis under IFN-λ regulation (Figure 2).

IFN-λ mediated regulation of intestinal inflammation

As a hallmark of intestinal inflammation in COVID-19 patients who have diarrhea, neutrophils are rapidly recruited from the blood after IEC infection. Neutrophils also restrict pathogen invasion through phagocytosis, reactive oxygen species (ROS), and the degranulation and release of cytotoxic antimicrobial molecules[85]. In contrast, excessive recruitment of neutrophils promotes inflammation. Thus, it is important to control the recruitment of neutrophils. Published studies show that IFN-λ provides a better balance of antiviral protection, with minimal inflammation and tissue damage. Specifically, IFN-λ in neutrophils inhibits the activation and phosphorylation of kinase AKT, which prevents the assembly of nicotinamide adenine dinucleotide phosphate oxidase complex NOX2 and decrease ROS in a JAK2-dependent way[86]. The effects of IFN-λ in neutrophils are independent of protein synthesis and transcription, only depending on the activation of AKT being inhibited[87]. Another study showed that human neutrophils optimally respond to IFN-λ in vivo, potentially inducing ISG levels to rise during inflammatory conditions by up-regulating IFN-λR1[88]. Mutual regulation between IFN-λ and neutrophils promotes intestinal resistance to SARS-CoV-2 invasion, and reduces the damage of intestinal tissue caused by inflammation.

CONCLUSION

IFN-λ potentially has a protective role in COVID-19 patients with intestinal infections. However, there is increasing evidence that IFN-λ has a complex dual role in the intestinal tract. Exposure to IFN-λ might increase intestinal permeability, which is associated with the disruption of junctional proteins[89]. Elevated IFN-λ is also related to lower numbers of Paneth cells and increased apoptosis of epithelial cells in the small intestinal[90]. In contrast, IFN-λ has been reported to exhibit an antiviral effect in the intestinal tract, and promotes the healing of IECs[71-73]. Although IFN-λ has an anti-SARS-CoV-2 effect in the intestinal tract, the mechanism remains unclear. Furthermore, how IFN-λ affects other intestinal cells (Paneth cells, goblet cells, and ISCs) and tight junctions in the intestine of SARS-CoV-2 patients requires investigation. Although IFN-λ has strong potential for use in COVID-19 intervention strategies, the mechanisms need to be elucidated first.

FOOTNOTES

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MINIREVIEWS

Comprehensive review on small common bile duct stones

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Abstract

Common bile duct stones are among the most common conditions encountered by endoscopists. Therefore, it is well researched; however, some items, such as indications for endoscopic papillary balloon dilatation (EPBD), safety of EPBD and endoscopic sphincterotomy in patients receiving dual antiplatelet therapy or direct oral anticoagulant, selection strategy for retrieval balloons and baskets, lack adequate evidence. Therefore, the guidelines have been updated with new research, while others remain unchanged due to weak evidence. In this review, we comprehensively summarize the standard methods in guidelines and new findings from recent studies on papillary dilation, stone retrieval devices, difficult-to-treat cases, troubleshooting during the procedure, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture.

Key Words: Choledocholithotomy; Choledocholithiasis; Common bile duct stones; Endoscopic papillary balloon dilation; Endoscopic sphincterotomy; Small common bile duct stones

Core Tip: In this review, we comprehensively summarized the standard methods for patients with small common bile duct stones in guidelines and new findings from recent studies on papillary dilation, stone retrieval devices, difficult-to-treat cases, troubleshooting during the procedure, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture.

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INTRODUCTION

Cholangitis is the second or third most common cause of community-acquired bacteremia, with common bile duct (CBD) stones being the most common[1,2]. Recurrence of CBD stones is common, with 111 (11.3%) of 983 patients who underwent endoscopic sphincterotomy (EST) recurred during a median follow-up of 7.5 years, and the cumulative recurrence rates at 5, 10, 15, and 20 years were 8.5%, 12.5%, 19.1%, and 24.2%, respectively[3]. It is frequently encountered by endoscopists, and it is important to improve short-term outcomes and prevent the long-term recurrence of cholelithiasis. This review focuses on small CBD stones. Although the international definition of small CBD stones has not been established, we have followed the standard of approximately 10 mm in some studies[4,5]. We described papillary dilation, stone extraction, difficult cases, troubleshooting during stone extraction in small CBD stones, and complicated cases of cholangitis, cholecystolithiasis, or distal biliary stricture and summarized the European, American, and Japanese guidelines. Moreover, this review addressed the novel literatures on endoscopic papillary balloon dilatation (EPBD) dilation times to prevent post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP)[6], the duration of direct oral anticoagulants (DOAC) and dual antiplatelet therapy (DAPT) withdrawal to safely perform EST[7,8], EST with balloon dilation (ESBD), and the comparison of the effects of retrieval balloon and basket catheters for small CBD stone extraction[9,10].

COMPARISON OF EPBD AND EST

Papillary dilation is divided into EST and EPBD, and a nationwide administrative database of 61000 hospitalized patients with CBD stones throughout Japan reported that EST was performed in 89% of patients and EPBD in 11%[11]. Knowledge of the success rate of CBD stone removal and the incidence of short- and long-term complications is important when deciding between EST and EPBD.

Success rates of CBD stones clearance

A meta-analysis reported that EPBD has a lower incidence of total clearance of CBD stones and more frequent lithotripsy basket use than EST[12]. However, 11 of 14 references in this study included cases of CBD stones larger than 10 mm. Conversely, there were no significant differences in total clearance of CBD stones in another meta-analysis by Liu *et al*[13].

Yu *et al*[6] reported that both EST and EPBD have obvious effects in the treatment of bile duct stones with minor diameters (< 10 mm) and small numbers (< 3). The EPBD balloons used in that study were mostly 8 and 10 mm in diameter, especially those with 8 mm in diameter. Because a typical papillary dilation balloon is 8 mm in diameter, the indication for EPBD may be CBD stones up to 10 mm in diameter, considering the flexibility of the papillae. However, even for CBD stones > 10 mm, EPBD combined with endoscopic mechanical lithotripsy may have a success rate of stone retrieval comparable to that of EST[13]. Therefore, EPBD may be useful in cases of coagulopathy in which CBD stones are larger than 10 mm.

There is lack of evidence for the possibility of very small stone extraction without EST or EPBD. It has been reported that if ESWL results in stone fragment size of 3 mm or less, there is a likelihood that the stone will be spontaneously discharged without EST. Therefore, it is possible that stone extraction can be performed without EST or EPBD if the size is less than approximately 3 mm[14], however, there are no studies that have directly examined this issue. Therefore, in principle, EST and EPBD are recommended for stone extraction of CBD stones, as recommended by the European Society of Gastrointestinal Endoscopy (ESGE) and Japan Gastroenterological Endoscopy Society (JGES) guidelines; however, it is at the endoscopist's discretion whether to perform stone extraction without these procedures for very small stones[15,16].

Incidence of short-term complications

In cases of EPBD compared to EST, post-ERCP pancreatitis increased, bleeding decreased, and there was no significant difference in perforation or post-ERCP cholangitis. PEP and hemorrhage are likely to occur especially in approximately 10% and less than 0.1% of patients in the EPBD group, respectively; and in approximately 3% and 3% of patients in the EST group, respectively[12]. The total data in the meta-analysis has variation in the patient's background; however, it is consistent with that of a previous report[17].

PEP: PEP may be a short-term complication when selecting EST/EPBD. ESGE describes the following risk factors for PEP:

Patient-related definite risk factors include suspected sphincter of Oddi dysfunction, female sex, previous pancreatitis, and previous PEP. Procedure-related definite risk factors, such as difficult cannulation, pancreatic guidewire passage > 1, and pancreatic injection. Patient-related risk factors include younger age, non-dilated extrahepatic bile duct, normal serum bilirubin, absence of chronic pancreatitis, and end-stage renal disease. Procedure-related risk factors include precut sphincterotomy, pancreatic sphincterotomy, failure to clear bile duct stones, intraductal ultrasound, and biliary balloon sphincter dilation[18]. ESGE especially recommends prophylactic pancreatic stenting in selected patients at high risk for PEP (inadvertent guidewire insertion/opacification of the pancreatic duct and double-guidewire cannulation).

In a multicenter randomized control study, 117 patients with bile duct stones were treated with EPBD; after treatment, the incidence of pancreatitis among those patients reached 15.4%, and two patients died from post-treatment complications[19]. Incomplete dilation of the papilla, intramucosal bleeding, and local edema were considered the main causes of PEP due to EPBD. Conversely, several reports of randomized control trials or network meta-analyses suggested that there is no direct consequence between PEP risk and EPBD[20,21], and PEP usually occurs in the mild or moderate stage [12]. Recently, a network meta-analysis reported that 2 to 5 min of EPBD could decrease the incidence of PEP compared to short-term (< 2 min) EPBD. In addition, it was also reported to reduce PEP without increasing the occurrence of other early complications by extending the duration of balloon dilatation [6]. However, the underlying mechanism for this result remains unclear. A possible reason could be that the dilatation with a small diameter balloon or short duration could result in inadequate papilla expansion; thus, the common discharge channel for bile and pancreatic juice tended to be narrow after the operation[6]. That study did not examine EPBD longer than 5 min; however, another study found that 5-min EPBD increases PEP compared to EPBD of 0.5-3 min[22]. Although this is a study of EPBD combined with small-incision EST, it may be advisable to avoid EPBD for more than 5 min[22]. Therefore, we use a 2-3 min EPBD.

In recent years, diclofenac or diclofenac and sublingual nitrates have been reported to be useful for the prevention of PEP[23,24]. ESGE also recommends routine rectal administration of 100 mg of diclofenac or indomethacin immediately before ERCP in all patients without contraindications to nonsteroidal anti-inflammatory drug administration[18]. These methods were not available in 2004 when EPBD was abandoned by many endoscopists, especially in America, and combining such methods may reduce the incidence of PEP due to EPBD. Furthermore, EPBD may be even safer in Asians, as some race-based studies have shown no increase in PEP in Asian populations[12].

Bleeding: ESGE guidelines suggest that patients should be considered at increased risk of post-EST bleeding if at least one of the following factors is present: anticoagulant intake, platelet count < 50000/mm^3, cirrhosis, dialysis of end-stage renal disease, intraprocedural bleeding, and low endoscopist experience[18].

ESGE, JGES, and the American Society of Gastrointestinal Endoscopy (ASGE) guidelines treat antiplatelet medications almost similarly for EST/EPBD. DAPT is permitted in EPBD without drug withdrawal, whereas EST requires DAPT withdrawal. Withdrawal regimens are similar across guidelines, with thienopyridine requiring 5-7 day withdrawal and continuation of aspirin or cilostazol monotherapy[18,25,26]. However, each guideline treats anticoagulants in a slightly complex and different manner. Although it is necessary to evaluate the risk of embolism and procedural bleeding when antithrombotic agents are stopped in EPBD, warfarin can be continued if the PT-INR is within the therapeutic range. In EST, treatment with warfarin can be continued, whereas the PT-INR is within the therapeutic range in Japan and America. However, in Europe and America, it is recommended to discontinue warfarin 5 d before EST and replace it with heparin 2 d before EST, especially in patients at high risk of embolism in aortic or mitral valve replacement, atrial fibrillation, or any thromboembolic risk. Once hemostasis is confirmed, antithrombotic agents must be restarted postoperatively in America, the next day in Japan, and within 2 d in Europe. Warfarin should be resumed after the procedure, and heparin should be used in combination until the PT-INR returns to the therapeutic range[18,25,26]. However, it is difficult to summarize each country's guidelines accurately and concisely; therefore, please refer to each country's guidelines for details. In addition, in DAPT and DOAC, there is a paucity of evidence regarding the ability of guideline-guided withdrawal periods to prevent bleeding[7,8,25,27].

With regards to hemorrhage, Mirjalili and Stringer[28] identified 98 arteries near the major papilla and reported blood vessel distribution on endoscopy. According to their report, blood vessel distri-

bution in the 10 to 11 o'clock region was low at 10%-11%; thus, cutting in this region has a low risk of hemorrhage. The ESGE and Japanese EST guidelines have cited this article[16,18]. No trials have compared hemorrhage and perforation according to cutting direction; however, adding to the reports that bile ducts tend to run in the 11 to 12 o'clock direction in the papillary region, cutting in the 11 to 12 o'clock direction is considered safe, and thus recommended by Japanese EST guidelines[16].

Others: The superior sphincter extends to the bile duct on the lateral wall of the duodenum, and cutting beyond this area increases the risk of perforation. In relation to the papilla, it is believed that the superior margin of the papillary bulge coincides with the middle sphincter, which is considered the upper cutting limit (Figure 1). However, anatomical examinations may not necessarily be consistent with actual living bodies, and depending on the cutting direction, perforation can occur even if the superior margin of the papillary bulge is not reached; thus, due care should be exercised[16]. Moreover, there is no evidence comparing incision size to the incidence of procedural adverse events or therapeutic outcomes following EST[16].

The incidence of short-term cholecystitis after ERCP could be caused by resistance to initial antibiotics on admission[29], and the incidence of long-term cholecystitis and the recurrence of stones in CBD could be decreased by EPBD compared to EST[6,12]. EST causes significant damages to the Oddi sphincter, and post-EST sphincter dysfunction easily occurs[30]. Then, the reflux of intestinal contents such as digestive juices, food residue, and bacteria may increase the risk of biliary tract infection and stone recurrence[31,32].

To summarize the characteristics of EST and EPBD (Table 1), EST is superior in terms of PEP reduction and bile duct large stone retrieval, while EPBD is superior in terms of bleeding reduction, long-term cholecystitis, and bile duct stone recurrence. Based on these findings, we consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult.

ESBD: Ding *et al*[4] defined a tunnel from the distal bile duct to the papillary orifice as an extraction tunnel (SET). Based on the anatomical structure, the tunnel was divided into two segments, with the distal bile duct and the intradural portion of the sphincter of Oddi comprising the proximal segment, including the proximal ring, and the intraduodenal portion of the distal segment of the papillae, including the distal ring around the orifice. Conventional EST cuts the distal segment almost completely from the orifice to near the duodenal wall, EPBD extends the entire SET, and EST + EPBD (ESBD) shortens the SET by cutting the distal ring and extends the proximal ring. Therefore, this combination technique is suitable for accessing the wide opening of the SET from an anatomical perspective[4]. In this study, ESBD was reported to reduce the number of treatments for complete stone removal, procedure time, use of mechanical lithotripters, and bleeding rate, and the incidence of PEP was reported to be comparable to that of EST. It has been reported that a small incision did not increase the risk of bleeding compared with non-EST, which might be attributed to a lower chance of injury to the major vessel in the papillary roof[20]. ESBD limits EST to small incisions, which may be the reason for reduced bleeding after ERCP. In a network meta-analysis, ESBD tended to be superior to EST in terms of successful stone removal in the first endoscopic session, the need for mechanical lithotripsy, and the risk of bleeding or perforation. However, none of these variables showed statistical significance[20]. Thus, ESBD may be superior to EST in overall efficacy and short- and long-term complications, and ESBD may be recommended over EST in the future; however, there is insufficient evidence to recommend ESBD over EST. Therefore, to justify updating the current guidelines, researchers will require more evidence that ESBD is superior to EST in terms of overall efficacy[20] and that ESBD may reduce the long-term recurrence rate of bile duct stones[33]. At this time, it is up to each endoscopist to decide whether to perform ESBD or EST.

COMPARISON BETWEEN BALLOON AND BASKET CATHETER

A recent meta-analysis found that balloon catheters for cholelithiasis were superior to basket catheters for complete stone removal[9]. However, there are some limitations in the studies included in this meta-analysis. Three of the four studies included in the review were on small stones (≤ 10-11 mm), and three of these articles used a four-wire retrieval basket catheter. Four-wire retrieval basket catheters are less suited to retrieve small stones than an eight-wire retrieval basket catheters and retrieval balloon catheters. Therefore, we cannot conclude that the basket catheter is inferior to a balloon catheter in the case of small CBD stones[5,34-36]. One meta-analysis study only included these three studies, but its conclusions were similar to those of a previous meta-analysis[10]. Ozawa *et al*[5] reported that small stones (maximum diameter, 6 mm) are an independent risk factor for failed stone removal; in their study, the basket failed to grasp a small stone in eight cases, and in four of which, the stones were successfully removed after an exchange with a balloon catheter. Therefore, they suggested that a retrieval balloon catheter may be more appropriate than a basket catheter for removing small stones[5]. However, Ozawa *et al*[5] also used a four-wire basket.

Table 1 Short- and long-term complications of endoscopic papillary balloon dilatation/endoscopic sphincterotomy

	Short-term complications			Long-term complications	
	Bleeding	**PEP**	**Perforation**	**Cholecystitis**	**Recurrence of stones in CBD**
Incidence					
EST	3%	3%	Rare	EST > EPBD	EST > EPBD
EPBD	Less than 0.1%	10%	Very rare		
Prophylactic methods	Cessation of anticoagulant and antiplatelet agents referred to each country's guidelines; EST with cutting at approximately 11 o'clock direction	Diclofenac; Pancreatic stenting in selected patients at high risk for PEP; 2-3 min EPBD in patients with EPBD	There is no evidence comparing incision size, the incidence of procedural adverse events, and therapeutic outcomes following EST	We consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult	We consider EPBD in cases of small bile duct stones, bleeding tendency, young age, and even in surgically altered anatomy in which EST is difficult

CBD: Common bile duct; EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilatation; PEP: Post endoscopic retrograde cholangiopancreatography pancreatitis.

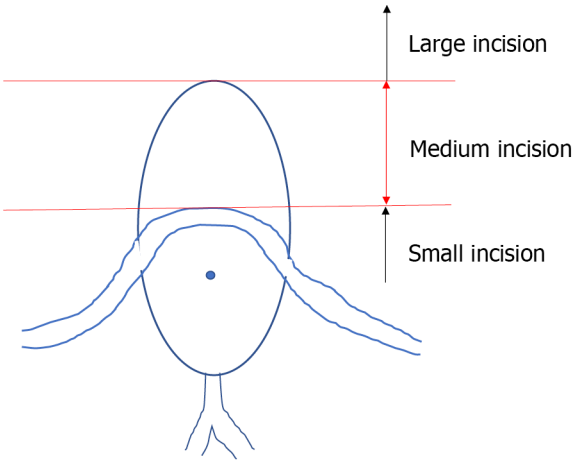

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Figure 1 The oral protrusion. Endoscopic sphincterotomy incision size. The risk of perforation increases when the incision exceeds the superior margin of oral protrusion.

Once a stone is captured in a basket, reliable extraction is usually ensured. More reliable traction associated with the basket catheter is cited as the main reason for its preferential use in Japan and Europe[5,9]. In the study by Ozawa *et al*[5], the balloon slipped past the stones and could not provide a sufficient traction force for stone extraction within 10 min in four patients in the balloon group, and the stones were successfully captured and withdrawn after exchange to the basket in all cases. However, a basket with a captured stone may occasionally become impacted at the papilla during extraction if the sphincterotomy is insufficient or if the stone is larger than estimated. According to the ESGE guidelines, the difference between balloon and basket catheters is slightly minimal, so endoscopists can use any of the two; meanwhile, according to the ASGE guidelines, the balloon catheter is highly recommended for safety issues related to basket impaction[18,37].

REMOVAL OF DIFFICULT SMALL BILE DUCT STONES

There are two main operations when retrieving CBD stones with a retrieval balloon or basket. First, the catheter was pulled with the right hand. The other is to apply right rotation and push on the endoscope and use the down angle with dial control, if necessary. The difference between the two is the direction of the force on the retrieval balloon or the basket. In the former, the retrieval balloon or basket faces the forceps hole at the endoscope tip, whereas, in the latter, they face the tip of the endoscope that is pushed in (Figure 2). The important basic rule is that the direction of the force applied to the catheter should coincide with the long axis of the bile duct, and one can choose the easier of the two methods to accomplish this.

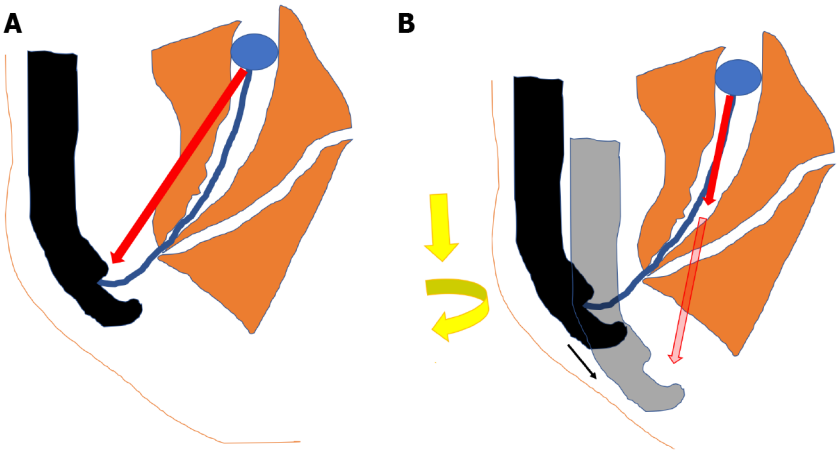

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Figure 2 Basket/balloon catheter operations. A: Direction of force on the retrieval balloon or basket when pulling the catheter with the right hand; B: Direction of the force on the retrieval balloon or basket when applying right rotation and pushing the endoscope.

However, in cases with pockets in the lower part of the bile duct, stone extraction is difficult. Once a stone is impacted at the corner pocket, the balloon passes alongside the stone without removing it, and stone removal is often difficult, even after repeated attempts. Such cases can be handled by pushing the stone up to the middle of the bile duct and then grabbing it with a basket or by using a basket shaped to extract the stone out of the pocket, such as a disposable NT retrieval basket (VorticCatch V: Olympus Medical Systems, Japan) (Figure 3).

Furthermore, stones near the bifurcation of the gallbladder duct are difficult to grasp using a retrieval balloon or basket (Figure 4). Surgery is considered in these cases; however, they can be addressed with cholangioscopy, such as when in conjunction with electronic hydraulic lithotripsy (EHL)[15]. When it is difficult to grasp a CBD stone, a basket that directly grasps the stone under cholangioscopy is available [38].

Enteroscopy-assisted ERCP (eERCP) is often difficult in cases of surgically altered anatomy (SAA). In cases of SAA, endoscopic ultrasound-guided transmural drainage (EUS-TD) or percutaneous transhepatic biliary drainage (PTBD) may be effective alternatives (Figure 5)[39].

In a multicenter retrospective cohort study involving 98 patients (49 EUS-TD and 49 eERCP groups), technical success was achieved in 98% of patients in the EUS-TD group compared to 65.3% of patients in the eERCP group (OR 12.48, $P = 0.001$). EUS-TD had a significantly shorter procedural time (55 *vs* 95 min, $P < 0.001$). However, more complications of mild/moderate severity occurred in the EUS-TD group (20% *vs* 4%, $P = 0.01$). The length of stay was significantly longer in the EUS-TD group (6.6 *vs* 2.4 d, $P < 0.001$)[40]. PTBD is also a useful alternative, with a reported success rate of approximately 97%, but this method of stone removal may cause problems, such as drainage tube trouble or an increased number of sessions[41].

TROUBLESHOOTING DURING STONE REMOVAL

A serious drawback of basket catheters is that during stone extraction, the basket with the captured stone is impacted in the lower bile duct or papilla. When basket impaction occurs, the basket must first be opened and pushed upwards into the hepatic hilum. An attempt was made to curl the basket wires back and disengage the stone (Figure 6). If this technique fails, more complicated techniques, such as mechanical lithotripsy and intra-extracorporeal lithotripsy, are required[5]. To use a lithotripter, such as BML-110A-1 (Olympus Medical Systems, Tokyo) (Figure 7), which can be retrofitted to a basket catheter, the basket catheter is cut outside the body, the endoscope is removed from the body, and the wires of the basket catheter from the mouth are wrapped around the lithotripter. However, if the basket cannot be unmated even with a lithotripter, a cholangioscope can be helpful. The basket and grasped stone were visualized under the cholangioscope and crushed by an EHL or YAG laser (Figure 8).

SPECIFIC SITUATION

CBD stones complicated with cholangitis

The Tokyo Guidelines 2018 (TG18) and ASGE suggest that bile duct stone removal following EST in a single session may be considered in patients with mild or moderate acute cholangitis[42,43]. However,

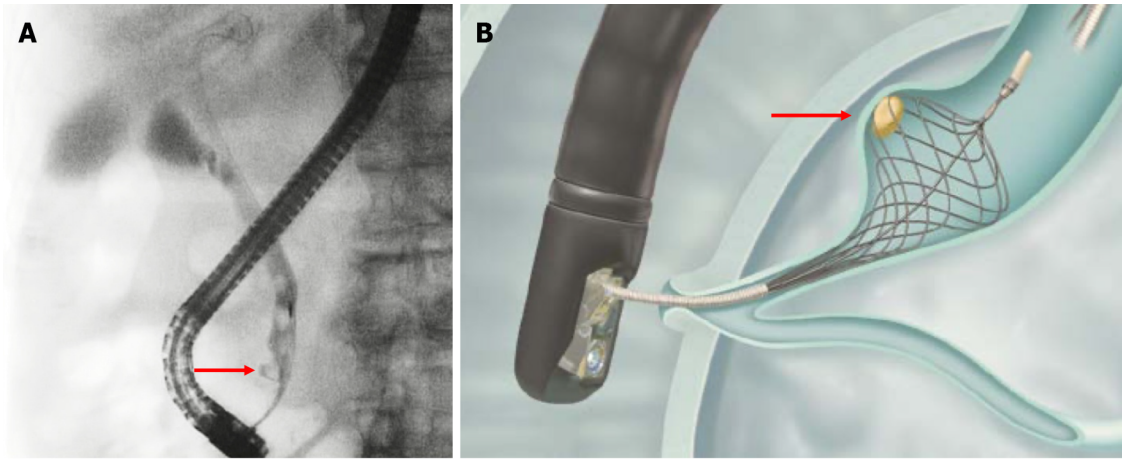

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Figure 3 Stone in the lower common bile duct pocket. Red arrows indicate a stone in the lower common bile duct pocket. A: A case with stone in the lower common bile duct pocket; B: Disposable NT retrieval basket (VorticCatch V: Olympus Medical Systems, Japan).

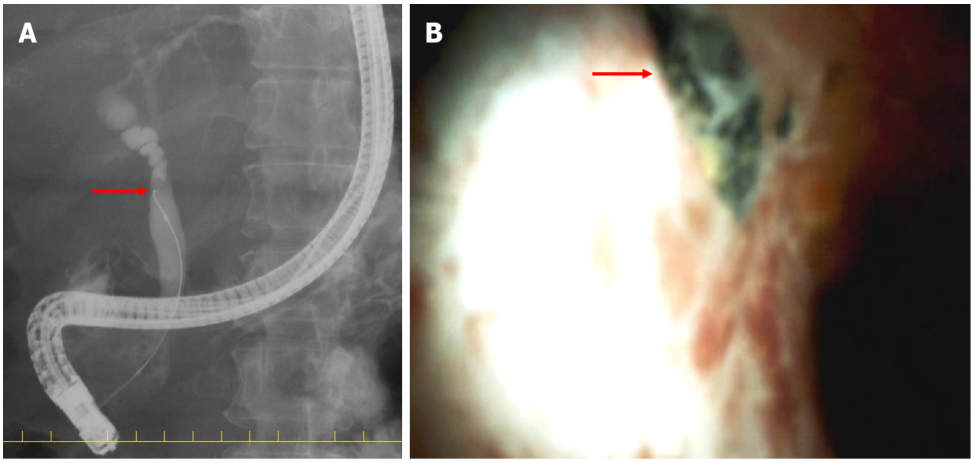

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Figure 4 Stone stuck in the bifurcation of the gallbladder duct. Red arrows indicate a stone stuck in the bifurcation of the gallbladder duct. A: The stone got stuck in the bifurcation of the gallbladder duct, as seen by endoscopic retrograde cholangiopancreatography. The guidewire could not be inserted into the gallbladder duct because of the obstruction by a stone; B: A stone stuck in the bifurcation of the gallbladder duct as observed by cholangioscopy.

given that hemodynamically unstable or coagulopathy patients might not tolerate procedural bleeding or adverse events, decompression alone should be considered in this group[42,43]. PEP does not increase even in cases of complicated cholangitis[43]. TG18 suggested that endoscopic nasobiliary drainage (ENBD) or endoscopic biliary stenting (EBS) may be considered for biliary drainage according to the patient's background and preference. It should be borne in mind that if patients experience discomfort from transnasal tube placement, they are likely to remove the tube themselves, particularly in elderly patients. EBS is an internal drainage technique that does not cause discomfort or loss of electrolytes or fluids. In contrast, ENBD is an external drainage technique that allows monitoring or washing of bile *via* the transnasal tube, particularly if the bile is purulent[42]. The ESGE did not provide any recommendations for these[15]. We present a table summarizing each guideline, focusing on key points (Table 2).

CBD stones complicated with cholecystolithiasis

In the general population, CBD stones complicated with cholecystolithiasis commonly occurs. The established gold standard for the treatment of symptomatic cholecystolithiasis is laparoscopic cholecystectomy (LC), but the treatment option for CBD stones is yet to be clarified. CBD stones complicated with cholecystolithiasis can be treated with two-session minimally invasive and one-session feasible strategies. The former requires pre- or post-LC ERCP, whereas the latter requires LC plus intraoperative laparoscopic CBD exploration (LCBDE) or LC with intraoperative ERCP[44]. As per efficacy, morbidity, or mortality endoscopic and surgical techniques for extracting these stones are equally suitable[45]. However, one-session procedures usually result in a shorter hospital stay[15].

Table 2 Key points of each guideline

	JGES/TG18	ESGE	ASGE
Papillary dilatation	EST is standard	EST is standard	EST is standard
	EPBD is determined by age, scheduled RFA, antithrombotic medications, parapapillary diverticulum, reconstructed bowel, and stone diameter and number	In anticoagulant users and in cases of SAA, EPBD is an option for stones smaller than 8 mm	In anticoagulant users, in cases of SAA, and in cases of intradiverticular papilla, EPBD is an option
Cases of antithrombotic agents use (EST, high bleeding risk procedures)	ASA or CLZ alone may be continued	ASA or CLZ alone may be continued	ASA or CLZ alone may be continued
	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ	Thienopyridines discontinued for 5-7 d or replaced with ASA or CLZ
	Warfarin may be continued if in therapeutic range; DOAC is withdrawn on the EST day only	Warfarin stopped 5 d ago and LMWH was started 2 d ago (LMWH also stopped 24 h ago). DOAC stopped 48 h ago	Warfarin users can be treated urgently if INR < 2.5. DOACs should be discontinued prior to treatment, with a discontinuation period of twice the half-life. Heparin replacement is recommended in patients at high risk of thrombosis
	Resumed the next day	Warfarin or DOAC resume within 48 h. Warfarin is used with LMWH until the optimal concentration is reached	Resume at the end of the procedure if hemostasis is confirmed. However, evidence for DOACs and APAs are scant
Stone retrieval	No superiority of balloons and baskets is noted	Efficacy of balloons and baskets is almost equal	Recommend using balloons rather than baskets
Complicated cases of cholangitis	Patients who are hemodynamically unstable, coagulopathic, or receiving antithrombotic agents; it was believed that decompression alone should be considered.EBS and ENBD are almost equal	Not stated	Given that hemodynamically unstable patients might not tolerate procedural bleeding or adverse events, it was believed that decompression alone should be considered in this group as well as for patients who are coagulopathic and/or are receiving antithrombotic agents and those who would need to have anticoagulation resumed immediately after sphincterotomy (*e.g.*, patients with mechanical heart valves)

APA: Antiplatelet agent; ASA: Acetylsalicylic acid; ASGE: American Society for Gastrointestinal Endoscopy; CLZ: Cilostazol; DOAC: Direct oral anticoagulant; EBS: Endoscopic biliary stent; ENBD: Endoscopic nasobiliary drainage; EPBD: Endoscopic papillary balloon dilation; ESGE: European Society of Gastrointestinal Endoscopy; EST: Endoscopic sphincterotomy; JGES: Japan Gastroenterological Endoscopy Society; LMWH: Low-molecular-weight heparin; SAA: Surgically altered anatomy; TG18: Tokyo guideline 2018.

Moreover, a recent meta-analysis has demonstrated that the one-session procedure has a higher success rate than the two-session procedure[46].

For one-session procedures, many surgeons prefer the less invasive and less complicated transcystectomy approach, however, bile duct incision is recommended for dilated CBD, large diameter and multiple stones, impacted stones, and stones with intrahepatic localization[47,48]. It is recommended to start with transcystectomy and move unto exploration by bile duct incision if difficult[44,49]. Laparoscopic stone removal can be performed fluoroscopically or cholangioscopically. The use of a flexible cholangioscope is the most preferred method because of its accuracy and direct visual control. However, one-session procedure requires advanced laparoscopic techniques, a long learning curve, and specialized equipment, and these qualities may not exist in all treatment facilities[50-52]. ESGE recommends that transcystic or transductal exploration of the CBD is a safe and effective technique for removal of CBD stones in patients undergoing laparoscopic cholecystectomy, provided that local expertise and resources are adequate[15]. It is of note that results of surgical treatment of CBD stones, which are generally excellent in published reports, are usually from laparoscopic centers of excellence, however, there are hardly reports by less experienced surgeons. Therefore, the ESGE does not clearly state whether one-session or two-session procedure should be preferred.

There are no recent reports on laparoscopic surgery for small CBD stones, however, Huang *et al*[53], in their report on laparoscopic surgery for small CBD with CBD stones, indicated that it is safe and feasible for small CBD patients to perform LCBDE.

CBS stones complicated with distal biliary stricture

Few reports have been published on CBD stones extraction with distal biliary stricture[54,55], however, plastic stent(s)[56,57], covered self-expandable metallic stent(s) (cSEMS)[56-58], balloon dilation[59], and surgery[60] have been used for dilating bile duct stenosis. However, balloon dilation carries the risk of

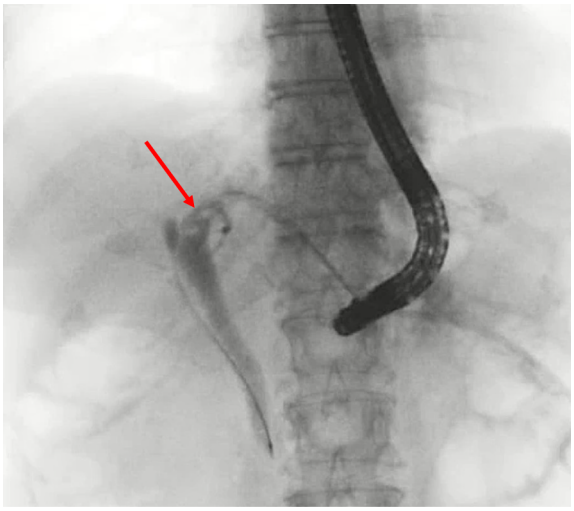

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Figure 5 Case of total gastrectomy with RY reconstruction. Enteroscopy-assisted endoscopic retrograde cholangiopancreatography was unsuccessful; therefore, an endoscopic ultrasound-guided hepaticogastrostomy was performed. The red arrow indicates bile duct stones.

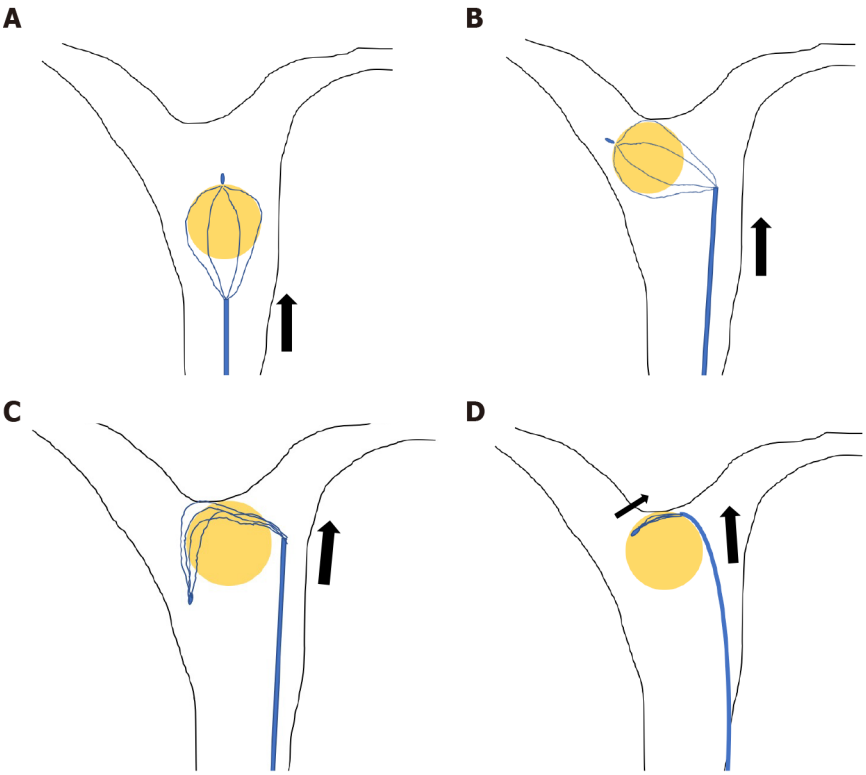

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Figure 6 Release of grasped stones. A: Push the basket catheter up into the hepatic hilum; B: Push further to invert the grasped stone; C: Push and deflect the basket wire; D: Close the basket while pushing the catheter.

bile duct injury. Therefore, when endoscopic stone extraction is performed for CBD stones with benign biliary stricture, it may be advisable to use multiple plastic stents or cSEMS for several months and perform endoscopic stone extraction after bile duct dilation is achieved[61]. Combining them with mechanical lithotripsy may also be useful[54]. Ogura *et al*[55] reported that transluminal stone extraction passing through the EUS-TD route, without passing through the distal bile duct might be useful. Reports of CBD stones with malignant biliary stricture are even more scarce, however, the safety of 6-8 mm balloon dilation for malignant biliary stricture has been reported[62]. In malignant biliary stricture with limited prognosis, stenting alone may be sufficient and stone extraction may not be necessary, however, balloon dilation for stone extraction may be considered in cases of short-term stent obstruction.

Figure 7 BML-110A-1 (Olympus Medical Systems, Tokyo). The authors have obtained the permission for figure using from the Olympus (Supplementary material).

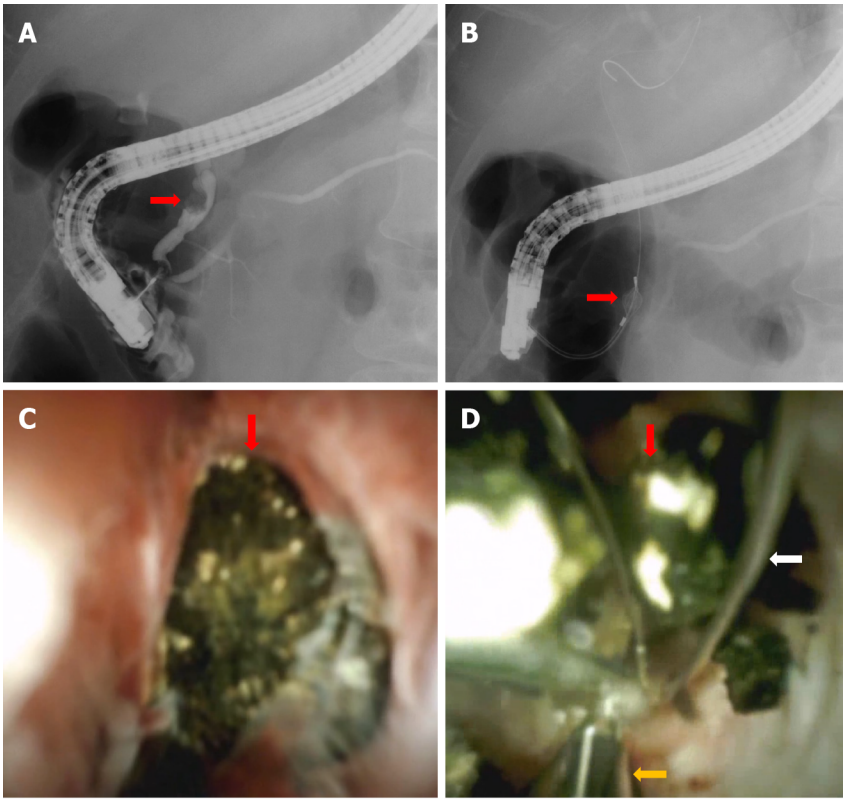

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Figure 8 The case in which the basket could not be unmated even with the lithotripter. The basket and grasped stone were visualized under cholangioscopy and crushed using electronic hydraulic lithotripsy (EHL). The red arrows indicate common bile duct (CBD) stones, white arrow basket catheter, and orange arrow EHL probe. A: CBD stone; B: CBD stone grasped by basket; C: CBD stone grasped by basket as seen by cholangioscopy; D: CBD stone crushed with EHL.

CONCLUSION

While EST is the standard treatment for papillary dilatation, EPBD is also a viable option for younger patients who wish to reduce the risk of long-term recurrence and patients with coagulopathy. EPBD is considered to have a lower risk of bleeding and perforation than EST. Several methods have been recently proposed to reduce PEP, the greatest weakness of EPBD. We would also like to focus on ESBD, which should be the subject of future research.

For small stones in the CBD, it is not necessary to strictly distinguish between the retrieval balloon and the basket; however, if one device cannot remove the stone, it is recommended to use the other. In cases of pockets in the lower bile duct, Voltic catch V is also useful. It is also important to gain experience in the use of EUS-TD, lithotripter, and cholangioscopy to deal with troubleshooting such as stones stuck in the basket and difficult cases of stone retrieval.

In cases of complicated cholangitis, stone retrieval can be performed in mild or moderate cases in a single session. In severe cases, decompression alone should be considered, and EBS is generally

recommended. Cases of CBD stones complicated with cholecystolithiasis that are scheduled for one-session surgical treatment or CBD stones complicated with distal biliary stricture should be treated in facilities with adequate experience and equipment.

FOOTNOTES

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MINIREVIEWS

Liver transplantation in the management of cholangiocarcinoma: Evolution and contemporary advances

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Abstract

Cholangiocarcinoma (CCA) is an aggressive malignancy arising from the biliary epithelium. It may occur at any location along the biliary tree with the perihilar area being the most common. Prognosis is poor with 5-year overall survival at less than 10%, typically due to unresectable disease at presentation. Radical surgical resection with clear margins offers a chance of cure in patients with resectable tumours, but is frequently not possible due to locally advanced disease. On the other hand, orthotopic liver transplantation (LT) allows for a radical and potentially curative resection for these patients, but has been historically controversial due to the limited supply of donor grafts and previously poor outcomes. In patients with perihilar CCA, within specific criteria and following the implementation of a protocol combining neoadjuvant chemoradiation and LT, excellent results have been achieved in the last decades, resulting in its increasing acceptance as an indication for LT and the standard of care in several centres with significant experience. However, in intrahepatic CCA, the role of LT remains controversial and owing to dismal previous results it is not an accepted indication. Nevertheless, more recent studies have demonstrated favourable results with LT in early intrahepatic CCA, indicating that, under defined criteria, its role may increase in the future. This review highlights the history and contemporary advances of LT in CCA, with particular focus on the improving outcomes of LT in intrahepatic and perihilar CCA and future perspectives.

Key Words: Cholangiocarcinoma; Klatskin tumor; Liver transplantation; Liver cancer; Liver resection; Neoadjuvant therapy

Core Tip: Cholangiocarcinoma (CCA) is an aggressive malignancy with poor prognosis. Radical surgical resection with clear margins may offer a chance of cure but is frequently not possible due to locally advanced disease. In the last two decades, within specific criteria, a protocolised combination of neoadjuvant chemoradiation and orthotopic liver transplantation (LT) has produced excellent results in patients with perihilar CCA, while favourable results have been shown with LT in early intrahepatic CCA in recent years. We review the history and contemporary advances of LT in CCA and discuss future perspectives.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignancy of the biliary epithelium. It is rare, affecting less than 6 persons per 100000 population[1,2]. CCA carries a poor prognosis with 5-year overall survival (OS) rates for all stages and subtypes at less than 10%[3-5]. CCA is further subdivided depending on the site of origin of the tumour in the biliary tract, with intrahepatic, perihilar (further subdivided by the Bismuth-Corlette classification) and distal variants recognised (Figure 1). These subtypes vary not only in their anatomical site, but also their biology with distinct phenotypes[6].

Complete surgical resection is the only curative therapy in CCA but is precluded in most individuals due to advanced disease at presentation. Unresectable disease has a median survival of between 6 mo to 1 year[7]. The type of surgical resection offered varies depending on the anatomic subtype of the CCA. Intrahepatic and perihilar CCAs mandate liver resections with or without excision of the extrahepatic bile ducts depending on the location and radiological[8] extent of invasion of the tumour. The risk of postoperative liver failure is largely dictated by the quality and volume of the future liver remnant which may prohibit resection in many patients. Techniques such as portal vein embolization and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) have been developed to extend the limits of resectability in liver surgery by inducing hypertrophy in the contralateral side of the liver (if/when disease-free), hence increasing the future liver remnant[9]. However, ALPPS is largely considered contraindicated in perihilar CCA (phCCA) owing to high perioperative mortality (48% at 90 d)[10]. For distal bile duct tumours, excision of the extrahepatic bile duct is combined with a pancreatoduodenectomy to achieve an adequate oncological margin.

With all of the above procedures, arteriovenous invasion of the tumour was historically seen as an absolute contraindication to resection with poor outcomes. More recently, both portovenous, caval and arterial reconstruction of hepatic, superior mesenteric and even the coeliac arteries have been described, with some series showing favourable long-term outcomes. Venous reconstruction can be performed with similar perioperative morbidity to conventional resection, whilst long-term survival is equivalent in some series[11]. Arterial reconstruction is associated with higher perioperative morbidity and mortality and significantly lower long-term disease-free and OS compared to standard resection, however, outcomes remain significantly superior to palliative care[12,13].

CCA may also involve both liver lobes either as a single mass at the hilum (Bismuth-Corlette type IV phCCA), as multicentric disease or by metastasis making disease unresectable without complete hepatectomy.

Despite this, most patients remain unresectable by virtue of locally advanced or distant metastatic disease, with a dismal median survival of 11.7 mo with palliative chemotherapy[7].

Liver transplantation (LT) has emerged as a potential solution to expand resectability of locally advanced CCA, with promising early patient outcomes. There is also new evidence that LT may confer superior patient outcomes even in technically resectable tumours[14]. This review aims to summarise the literature on the evolving role of LT in CCA.

HISTORY OF LIVER TRANSPLANTATION FOR MALIGNANCY

Initial indications for orthotopic liver transplants were focused on patients with malignancy[15]. Indeed, the second successful LT was performed for intrahepatic CCA (iCCA), by transplantation pioneer Thomas Starzl at the University of Colorado in 1963[15,16]. The recipient patient died on postoperative day 7 from respiratory failure and gastrointestinal bleeding. The early experience with LT unfortunately demonstrated prohibitively high perioperative mortality due to pulmonary emboli and haemorrhage, because of the veno-venous bypass and excessive anticoagulation used at the time. Coagulopathic

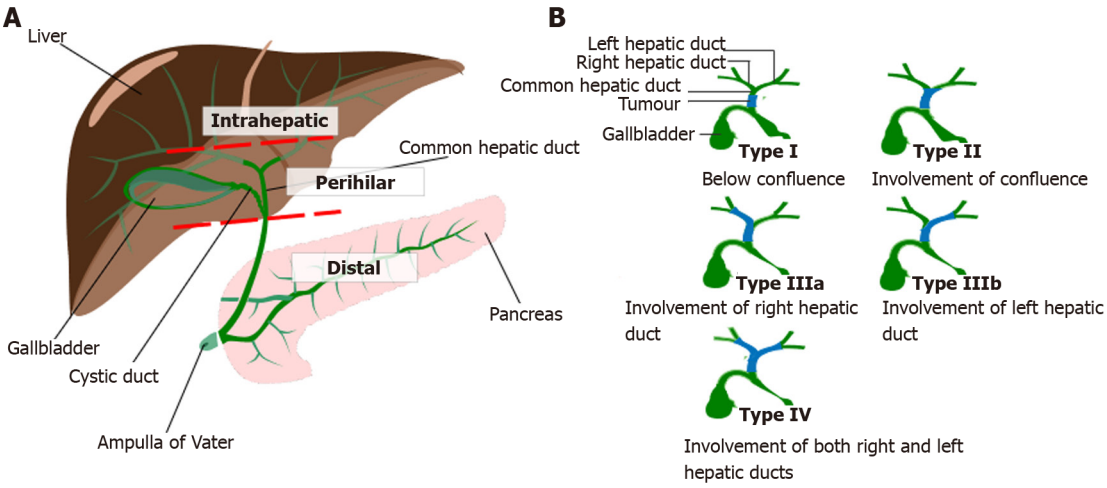

Figure 1 Classification of cholangiocarcinoma[62,71]. A: Anatomical classification of cholangiocarcinoma: Intrahepatic cholangiocarcinoma-proximal to second order bile ducts; Perihilar cholangiocarcinomas-between second order branches of right and/or left hepatic ducts and cystic duct confluence; Distal cholangiocarcinoma-between cystic duct confluence and Ampulla of Vater; B: Bismuth Corlette Classification of perihilar cholangiocarcinoma, https://creativecommons.org/licenses/by-sa/4.0/deed.en. Citation: Borakati A, Froghi F, Bhogal RH, Mavroeidis VK. Stereotactic radiotherapy for intrahepatic cholangiocarcinoma. *World J Gastrointest Oncol* 2022; 14: 1478-1489 [PMID: 36160742 DOI: 10.4251/wjgo.v14.i8.1478] and Wikimedia Commons. File: Bismuth corlette classification for perihilar cholangiocarcinomas.svg. 2020 Oct 9 [visited 3 February 2023]. Available from: https://commons.wikimedia.org/wiki/File:Bismuth_corlette_classification_for_perihilar_cholangiocarcinomas.svg.

complications were overcome with the routine use of thromboelastography to guide correction of clotting derangements in real-time. Veno-venous bypass was later used without anticoagulation and obviated by newer surgical techniques such as the 'piggy-back' method which retains the recipient inferior vena cava and does not necessitate complete clamping of this vessel and the resultant haemodynamic instabilities[17]. The other main driver of early postoperative mortality was biliary sepsis and obstruction. This was largely overcome by routinely performing biliary anastomoses over T-tubes or by using Roux-en-Y reconstructions.

The development of improved organ preservation solutions, most notably the University of Wisconsin solution in 1988[18], allowed greater cold ischaemic times with consequent reductions in vascular and biliary complications[19]. At present, liver malignancies indicated for LT in well selected patients include hepatocellular carcinoma (HCC), hepatic epitheloid hemangioendothelioma, hepatoblastoma and metastatic neuroendocrine tumours, while an increasing interest is developing about its role in CCA and metastatic colorectal cancer[20].

LIVER TRANSPLANTATION FOR CHOLANGIOCARCINOMA

These advances combined with use of newer cyclosporine and steroid immunosuppression allowed post-operative survival to increase significantly by the early 1980s and the Colorado group reported 1-year survival in excess of 70% during this time. This allowed meaningful evaluation of the oncological benefits of LT for the first time with postoperative recurrence rates and OS with cancer specific mortality. Of the 6 patients transplanted for unresectable CCA who survived more than 1 mo postoperatively, only 3 (50%) had recurrence free survival at 1 year. Those who did have recurrence all succumbed to disease at 1 year. The survival for those without recurrence at 1 year, ranged from 20 to 54 mo[21]. Poor survival for LT in CCA continued until the early 1990s, with 5-year overall, at less than 17% and recurrence greater than 50% in published series at the time[22-24].

The reasons for poor oncological outcomes despite optimisation of the LT procedure and immunosuppression protocols are in the main, twofold: Poor patient selection in early series, with presence of extrahepatic disease and utilisation for all anatomical subtypes of CCA, and absence of any neoadjuvant or adjuvant therapies to prevent recurrence.

This led to the development of neoadjuvant chemoradiotherapy and pre-transplant exploratory laparotomy protocols by the University of Nebraska[25] and Mayo Clinic in 1988 and 1993 respectively for phCCA only. The latter's results, initially published in 2000, showed of the 8 patients transplanted with follow-up > 12 mo, all were alive at their last follow-up (median 44 mo, range 17 to 83 mo) and only one patient developed a recurrence[26]. A subsequent study with 28 patients showed a 5-year actuarial OS rate of 82% with only 4 (14.2%) developing recurrence at 23 to 63 mo post-LT[27].

These impressive results have led to the adoption of the Mayo Clinic protocol more widely (Figure 2). In brief, patients with early stage hilar CCA on imaging (< 3 cm lesions, with no metastasis or lymph node involvement) who are technically unresectable undergo endoscopic ultrasound and fine needle

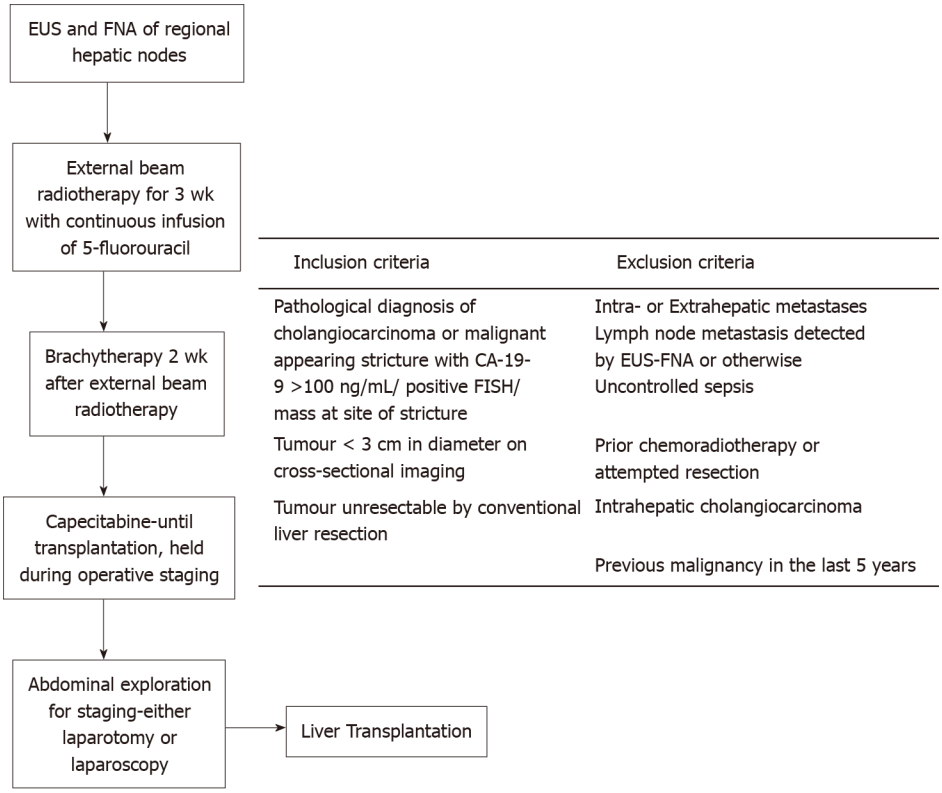

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Figure 2 Mayo clinic protocol for liver transplantation in hilar cholangiocarcinoma. EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; CA-19-9: Carbohydrate antigen 19-9; FISH: Fluorescence in situ hybridization.

aspiration of regional lymph nodes. Those with negative lymph nodes are given chemoradiotherapy for 5 wk and undergo laparoscopic or open abdominal exploration to exclude metastasis and examine regional lymph nodes prior to LT[27,28].

Current status of liver transplantation

There is a global discrepancy between donor livers availability and the number of patients on the liver transplant waiting list. Hence, there is understandably a clinical reticence in terms of utility to adopt LT for CCA given the historical poor prognosis when established benign and malignant indications for LT have proven significant benefit for this limited resource[29]. Established indications for LT include predominantly benign, chronic liver disease such as alcoholic and non-alcoholic fatty liver disease; autoimmune and metabolic pathologies such as primary sclerosing cholangitis (PSC) and Wilson's disease respectively and viral hepatitis. Paracetamol and other overdoses remain important acute causes of liver failure in Western populations which are also indications for LT. Among liver malignancies, the most widely established indication for LT is HCC, in which LT is only performed for early stage disease as outlined in the Milan Criteria[29]. Contemporarily 1- and 5-year OS for these indications following LT approach 95% and 85%, respectively[30]. New indications for LT will have to show comparable outcomes to the established indications to justify investment and allocation of donor livers for these purposes.

Following the publication of favourable results by the Mayo Clinic, with results similar to benign indications for LT, the United Network for Organ Sharing in United States, granted an exception to the Model for End Stage Liver Disease (MELD) used to prioritise those on the waiting list for LT. This exception allowed patients who had completed neoadjuvant therapy and had favourable operative staging to be added on the waiting list for LT with a MELD score equivalent to that of a patient with stage 1 HCC (as an established indication for LT)[31]. This improved access to LT for patients with phCCA and influenced the adoption of LT more widely with use of the MELD score and derivative scores internationally for LT allocations[29]. Further considerations of transplant allocation include the presence of underlying cirrhosis and greater degree of decompensation which increase the MELD score and may influence waiting time for transplantation but may also influence OS and recurrence rates when transplant is done for CCA.

The quality of the donor liver and whether it was from a live or deceased donor also has an impact on postoperative outcomes. Living donor LT may offer shorter waiting times, but is associated with higher rates of biliary complications (34% *vs* 17%, $P < 0.001$)[32]. Extended criteria donors with advanced age, steatosis or donors after circulatory death (DCD)[33] may increase the pool of organs but may be

associated with higher graft failure rates of 27.3% at 3-year with DCD livers *vs* 18.2% with donation after brainstem death[34]. Normothermic machine perfusion is another new technique which allows evaluation of graft function preoperatively and may further expand the donor pool and improve outcomes by selecting grafts with more objectively proven function[35].

PERIHILAR CHOLANGIOCARCINOMA

The Mayo Clinic group published their series of LT for phCCA from the inception of their protocol in 1993 to 2019. Of 376 patients included into the protocol, 234 (62%) ultimately proceeded to LT. The reasons for not proceeding to LT were primarily due to progression of disease prior to LT or metastasis found on staging surgery. OS at 5 years [Kaplan-Meier (KM)] was 68% +/- 3% and at 10 years was 60% +/- 4%[36]. Other long-term follow-up and larger series have validated the Mayo Clinic protocol for LT in phCCA. A multi-centre retrospective study from the United States of patients undergoing LT for phCCA, with any neoadjuvant chemo- or radiotherapy reported on 287 patients. Two hundred and fourteen (74%) proceeded to LT. Actuarial OS and disease-free survival (DFS) were 78% and 80% respectively at a median follow-up of 2.5 years. KM 5-year DFS was 65% [95% confidence interval (CI) 57%-73%] with 5-year OS at 53% (95%CI: 46%-60%)[37]. Multicentre series in the United Kingdom[37], Spain[38] and Germany[39] showed poorer results, likely as neoadjuvant protocols were not adopted at the time. Hidalgo *et al*[37] in the United Kingdom reported 5-year actuarial OS of 20%, Robles and colleagues 5-year OS of 30% in Spain and 31% in Germany pre-1998. Post-1998, when neoadjuvant chemotherapy was adopted in German protocols, 5-year OS after LT increased to 48%. A recent meta-analysis of retrospective series published before 2019 reported on 428 patients who underwent LT for phCCA and demonstrated pooled 5-year OS without neoadjuvant therapy of 31.6% (95%CI: 23.1%-41.7%) whilst with neoadjuvant therapy OS was 51.7% (95%CI: 33.8%-69.4%). Three years recurrence rates were 51.7% (95%CI: 33.8%-69.4%) without neoadjuvant therapy *vs* 24.1% (95%CI: 17.9%-30.9%), highlighting the importance of neoadjuvant regimes[40].

Comparison of LT to resection with curative intent for phCCA has shown a trend in favour of LT, albeit with high heterogeneity between studies. Ethun *et al*[14] performed a registry-based study of patients with phCCA at 3 United States centres, who underwent either curative intent liver resection or LT. Patients undergoing LT had neoadjuvant regimens similar to the Mayo protocol. One hundred ninety-one patients underwent curative intent resection (without neoadjuvant therapies), whilst 46 underwent LT. Five-year KM OS was 64% in the LT *vs* 18% for the resection group ($P < 0.001$). There was no significant difference in actuarial recurrence at a median follow-up of 23 mo, with 24% *vs* 37% ($P = 0.19$) for LT and resection groups respectively. Twenty nine percent of the patients undergoing LT had no residual disease on explant, suggesting complete response to neoadjuvant therapy. The authors also conducted an intention-to-treat analysis of patients who were enrolled in the transplant protocol and patients who were planned for curative intent resection or who had attempted curative intent resection. Five-year OS was still higher in the LT group at 53% *vs* 17% ($P < 0.001$) for the resection group[14].

Moris and colleagues performed a meta-analysis of studies with patients with locally advanced phCCA comparing LT and resection with curative intent. Local staging and nodal involvement were similar in both groups. There was a higher rate of R0 resection in patients with LT, 92.2% *vs* 73.3% [Risk Ratio (RR) 1.17, 95%CI: 1.03-1.33, $I^2 = 46\%$]. They also reported a higher 3-year OS with a lower hazard ratio (HR) of death (HR 0.61, 95%CI: 0.4-0.93, $I^2 = 39\%$). At 5 years, there was a trend towards increased OS for LT, but this was not significant (HR 0.67, 95%CI: 0.44-1.02, $I^2 = 54\%$)[41].

PSC

PSC is an important subgroup of patients undergoing LT in CCA. PSC is a significant risk factor for development of CCA and is in itself an indication for LT due to progressive liver dysfunction. PSC is associated with a higher rate of multifocal CCA precluding oncological resection or necessitating more extensive resection. Further, the poor quality of the liver parenchyma due to the disease may mean that the regenerative capacity of the liver is reduced which in turn limits the extent of resection possible. These factors typically preclude curative liver resection, even for small, localised lesions[43]. This prompted consideration for the alternative approach of LT for CCA, in particular phCCA, to mitigate the limitations of standard liver resection, and is one of the accepted inclusion criteria for the Mayo Clinic protocol[27]. Current data are clearly in favour of a protocolised approach with neoadjuvant chemoradiation followed by LT in PSC-associated phCCA, as the optimal treatment strategy, with superior outcomes when compared with de novo phCCA[42-44]. The Mayo Clinic series found a significantly higher 5- and 10-year survival in patients with PSC after LT, 74% and 67% *vs* 58% and 47% respectively in patients with de novo CCA[35]. The meta-analysis by Cambridge *et al*[40] also supports that in unresectable phCCA, combined neoadjuvant chemoradiation followed by LT confers long-term survival in highly selected patients able to complete the protocol with the most favourable outcomes observed in PSC patients[43]. Despite the lack of direct evidence, it has been hypothesized that the significantly better outcomes in PSC patients could be at least in part explained by a possible higher responsiveness to radiation therapy for phCCA arising on a background of PSC[35,42,45].

INTRAHEPATIC CHOLANGIOCARCINOMA

While phCCA is now increasingly accepted as an indication for LT using the Mayo Protocol, iCCA is still largely considered a contraindication[30]. Although early attempts at LT for CCA did not distinguish between subtypes in many cases and consequently iCCAs were transplanted[21] the outcomes remained poor until the advent of neoadjuvant protocols, such as that of the Mayo Clinic which are only applied to phCCA. Most literature on LT, therefore, focuses on incidentally found iCCA on explant, in patients who were transplanted for presumed HCC, which does not typically undergo neoadjuvant systemic therapy prior to transplant but rather only locoregional therapy such as radiofrequency ablation. There remains a lack of evidence to support such protocols for iCCA, partly because iCCA is the least common subtype of CCA (10% *vs* phCCA which represents 50%[46]) but also because of its distinct biological characteristics compared to other forms of CCA, with some being defined as mixed hepatocellular and intrahepatic cholangiocarcinoma[2].

Krasnodębski *et al*[47] provided one of the oldest series of 8 patients found on explant to have iCCA, having undergone LT without neoadjuvant therapies, from 1994-2019. Five-year OS and DFS were 25% and 28.6% respectively[47]. Lee *et al*[48] reported on their experience of LTs with pre-transplant diagnoses of HCC who were found to have iCCA on explant pathology. The majority of these patients also underwent neoadjuvant locoregional therapies such as radiofrequency ablation or transarterial chemoembolization. Seventeen cases of iCCA were identified from 1998-2018 and had 5-year actuarial OS of 51.9% and recurrence rate of 29.4%. This compared to the patients with HCC transplanted contemporaneously with OS of 71.5% and recurrence rate of 10.8%. Mixed hepatocellular and CCA LTs had a similar OS to iCCA at 55.0% but a significantly higher recurrence rate of 40.7%[48]. It was thought that perhaps the poor results for iCCA were due to poor patient selection as with phCCA and HCC. The latter is established as an indication for LT, but widely governed by the Milan criteria which restrict transplantation to early-stage tumours[49].

Sapisochin and colleagues performed a multicentre retrospective cohort study in Spain comparing incidentally found iCCA/mixed HCC/iCCAs on LT explant and LT for HCC. They found a significant decrease in survival in patients with pure iCCA compared to HCC (5-year actuarial OS 51% *vs* 93% respectively, $P < 0.001$), however mixed HCC/iCCAs had no significant difference in 5-year actuarial OS at 86% ($P = 0.9$). Subgroup analysis of patients with uninodular tumours < 2 cm in size, showed that of 12 patients with mixed HCC/iCCA ($n = 5$) and pure iCCA ($n = 7$), 5-year actuarial OS was 62% *vs* 80% in the HCC group ($P = 0.4$), implying no significant difference in survival. This finding may have been due to the low numbers of patients however[50].

A follow-up international multicentre retrospective series evaluating LT in early stage iCCA with unresectable unifocal tumours < 2 cm in size was conducted. Five-year actuarial OS in 14 patients with early stage iCCA was 65% *vs* 45% in those with advanced disease. KM recurrence rates were 18% at 5 years for early tumours compared to 61% for advanced tumours[51]. These figures compare favourably to curative intent liver resections with negative margins in iCCA with 5-year OS in the range of 30%-40% for all sizes of tumour. Early stage tumours < 2 cm in size can have near 100% survival at 5 years with negative margin resections. However, recurrence remains high with 5-year recurrence as high as 80% in the literature[52,53]. Hue *et al*[54] also did not find any significant differences in survival or recurrence with resection or LT for iCCA in their propensity matched registry-based study in the United States[54].

Studies which include neoadjuvant therapy prior to LT similar to the Mayo Protocol are limited to small case series. Lunsford *et al*[55] evaluated 12 patients with non-metastatic iCCA > 2 cm in size who were unresectable. They underwent neoadjuvant gemcitabine and platinum-based chemotherapy for 6 mo, and those who showed stable disease or regression proceeded to transplant. Six (50%) proceeded to LT and 5-year KM OS was 83.3% (95%CI: 27.3%-97.5%). Three patients developed recurrent disease at a median of 7.6 mo[56]. Wong *et al*[57] performed a similar study with the addition of transarterial chemoembolization and pre-transplant operative staging. Of 18 patients who started the neoadjuvant therapies only 5 (27.8) proceeded to transplant. Follow-up was limited to 1 year, actuarial OS was 80% (the single death was due to tumour recurrence) and recurrence developed in 2 patients (40%)[56].

A meta-analysis combining the above studies and others found a pooled 5-year OS of 42% (95%CI: 29%-55%) and 5-year DFS of 49% (95%CI: 41%-57%). However, the studies were all clinically heterogeneous with different preoperative protocols, different stages of tumours, degrees of cirrhosis in the background liver and used a variety of donor grafts. There was also statistically significant heterogeneity in all the meta-analyses with I^2 values being significant at the < 0.01 Level[57].

Interpretation of all the above results is difficult given significant clinical differences in each study. But overall, it can be concluded that while LT can improve outcomes compared to palliative therapy for unresectable iCCA, the outcomes compared to transplant for other indications including phCCA are poorer at present, although evidence is limited. Very early stage unresectable tumours < 2 cm in size appear to have excellent prognosis with LT, with improved outcomes compared to even curative intent resection for iCCA. More evidence is needed, however, there is increasing opinion that unresectable iCCA may become an extended indication for LT in the same way as phCCA has. In 2020, the European Network for the Study of CCA endorsed the value of this modality as a potentially curative option for iCCA in a consensus statement, and recommended it should be considered especially in patients with

very early stage unresectable tumours (≤ 2 cm) and concomitant cirrhosis[2].

A summary of key series of liver transplantation for phCCA and iCCA is presented in Table 1.

DISTAL CHOLANGIOCARCINOMA

phCCAs may extend down into the distal bile duct and it is established with the Mayo Protocol to potentially treat these with combined LT and pancreatoduodenectomy and many of the studies discussed above incorporate this either as a planned procedure or due to intraoperative findings of tumour extension[40]. Literature on LT in distal CCA (dCCA) is limited as these tumours are less likely to involve the liver and can be completely excised with pancreatoduodenectomy or bile duct excision with reconstruction depending on the site of the tumour. Extension into the liver or hilum would typically render the disease unresectable. Attempts at hepatopancreatoduodenectomy for these patients have been technically challenging with high rates of perioperative morbidity and mortality at up to 97.4% and 26% respectively in recent series. Five-year OS has ranged from 17.9%-49.2%[58]. Total hepatectomy, LT and pancreatoduodenectomy may have potential to be a better alternative therapy in this population. Case reports are sparse as dCCA is commonly an incidental finding in the context of LT for phCCA. Sutcliffe *et al*[59] report two patients at King's College Hospital, United Kingdom who underwent LT for PSC but were found to incidentally have dCCA on the bile duct explant. They then underwent pancreatoduodenectomy 2 mo post-transplant. One patient died due to other causes with no evidence of recurrent disease over 5 years later. The other patient died 5 mo after pancreatoduodenectomy due to recurrence[59].

Patients with PSC may also develop dCCA and require resection for this and also LT for their underlying liver disease. Stauffer *et al*[61] reported on 6 patients with dCCA and PSC who underwent both LT and classical Whipple's procedure. One patient survived to 58 mo of follow-up without any evidence of recurrence. Another patient, with an early T1 tumour survived to 52 mo of follow-up, with recurrence. All other patients developed recurrence and did not survive to 2 years[60].

DIRECTIONS FOR FUTURE RESEARCH

The literature at present consists mainly of retrospective case series of LT in CCA and predominantly in phCCA. The studies are heterogeneous in patient populations, in terms of staging of disease, preoperative neoadjuvant therapies, types of donor livers used and postoperative management and follow-up. Prospective multi-centre observational studies are needed for more robust evaluation of the Mayo Protocol and any modifications to the protocol should ideally be evaluated in a randomized trial. Unfortunately, due to the many confounding variables involved in LT and CCA and the rarity of suitable cases in general, recruitment to such trials with restrictive inclusion criteria to keep populations homogenous will no doubt be challenging.

The TRANSPHILL trial in France, has been ongoing since 2014 and aimed to recruit 54 patients with resectable phCCA for randomisation to either curative intent resection or LT. The primary outcome is 5-year OS, and 3-year recurrence is a secondary outcome[61]. Results are eagerly awaited from this trial as of writing, however, if the positive results of Ethun *et al*[14] are confirmed, this would represent a true paradigm shift away from resection to LT in phCCA.

The development of international registries may allow more generalisable and homogenous research beyond simple case series in future, by increasing the number of cases presented and capturing all relevant variables systematically.

Adjuvant and neoadjuvant protocols

Addition of newer neoadjuvant therapies such as stereotactic body radiation therapy (SBRT) have shown promise in preventing disease progression with lower toxicity than traditional external beam radiotherapy and may be beneficial prior to LT[62]. Oncological therapy has advanced in the management of CCA with improvement of survival with combination of folinic acid, fluorouracil, and oxaliplatin (FOLFOX) as a second line chemotherapy agent[63] and with recent trial results showing a benefit for immunotherapies in CCA[64]. Both have potential as neoadjuvant or adjuvant therapies in CCA patients considered for LT. Finally, the BILCAP trial established capecitabine as an effective adjuvant agent after resection of CCA as the standard of care, by showing improved OS[65]. There have been no studies on adjuvant therapy after LT for CCA and this too may improve survival in this setting.

Further, immunosuppression is known to be a risk factor for the development of malignancy and recurrence. There has been little research into the optimum suppressive regimen to balance the risks of graft rejection *vs* risk of recurrence after LT for CCA. In LT for HCC, there has been some evidence that reduction of immunosuppression[66] and use of mechanistic target of rapamycin (mTOR) inhibitors of suppression has a beneficial effect on OS after recurrence[67]. A recent series of patients undergoing LT for iCCA and phCCA showed that a reduced immunosuppressive regimen after recurrence was

Table 1 Summary of key contemporary series of liver transplantation for cholangiocarcinoma

Ref.	Country	Design	Tumour anatomical subtype	Treatments (s)	Total patients (*n*)	Median follow-up/yr (range)	Outcomes (5-year)[1]		
							Overall survival (%)	Disease free survival (%)	Graft survival (%)
Azad *et al*[28], 2020	United States	Retrospective, single centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	De novo: 148, PSC: 228	-	De novo: 58 KM, PSC: 74 KM	De novo: 55 KM, PSC: 78 KM	-
Darwish Murad *et al* [36], 2012	United States	Retrospective, multi-centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	287	2.5 (0.1-17.8)	53 KM	65 KM	60 KM
Hidalgo *et al*[37], 2008	United Kingdom	Retrospective, single centre	Perihilar	LT	12	1.81	41 KM	-	-
Robles *et al*[38], 2004	Spain	Retrospective, multi-centre	Perihilar	LT	36	-	64	47	-
Ethun *et al*[14], 2018	United States	Retrospective, multi-centre	Perihilar	Neoadjuvant chemo-radiotherapy and LT	70	4.83 (0.025-10.6)	64 KM	24 KM	-
Krasnodębski *et al* [47], 2020	Poland	Retrospective, multi-centre	Intrahepatic	LT	8	30	25	28.6	-
Lee *et al*[48], 2018	United States	Retrospective, single centre	Intrahepatic	Neoadjuvant TARE/TACE/RFA and LT	17	Mean 4.2	51.9	70.6	-
Sapisochin *et al*[50], 2014	Spain	Retrospective, multi-centre	Intrahepatic	Neoadjuvant ethanol injection/TACE/RFA and LT	27	4.99 (0.97-11.9)	51	36	-
Sapisochin *et al*[51], 2016	International	Retrospective, multi-centre	Intrahepatic- early[1] and advanced	Neoadjuvant ethanol injection/TACE/RFA and LT	Early[1]-15, Advanced-33	Early-4.78, Advanced-2.06	Early-65, Advanced-45	Early-86.7, Advanced-48.5	-
Hue *et al*[54], 2021	United States	Retrospective, multi-centre	Intrahepatic	Neoadjuvant chemo-radiation and LT	74	3.9	33 KM	-	-
Lunsford *et al*[55], 2018	United States	Prospective, single centre	Intrahepatic	Neoadjuvant chemotherapy and LT	6	3 (2.42-4.25)	83.3	50	100
Wong *et al*[56], 2019	United States	Prospective, single centre	Intrahepatic	Neoadjuvant chemotherapy and TACE and LT	5	1.84	80	60	-

[1]Early tumours defined as < 2 cm in size.

PSC: Primary sclerosing cholangitis; KM: Kaplan-Meier; LT: Liver transplant; TARE: Trans-arterial radioembolization; TACE: Trans-arterial chemoembolization; RFA: Radiofrequency ablation.

significantly associated with survival with an increased odds ratio of survival at 4.2 (95%CI: 1.3-13.6; P = 0.02)[68].

Further advances in LT and perioperative care along with novel chemotherapeutic and biological agents may lead to further improved outcomes[69].

Establishment of LT as a treatment modality for CCA

For LT to become uniformly established as a treatment modality for CCA, in both iCCA and phCCA, consistent reports with 5-year survival exceeding 50% and in the range of established indications of LT are needed in each group. The supply of donor livers will need to be improved to match increased demand from this population of patients and technologies such as normothermic machine perfusion and policy changes such as opt-out organ donation[70] being more widely enacted are expected to increase this supply.

CONCLUSION

LT has emerged as an effective treatment option for CCA in suitably selected patients. Treatment of unresectable phCCA has been transformed by LT with all modern series approaching parity with outcomes with other indications for transplant. Early evidence shows that LT may offer even better survival than curative intent resection for early resectable hilar tumours and may render transplant as the treatment of choice for the disease. Evidence for LT in iCCA is limited, but has considerable potential, with comparable outcomes to perihilar tumours in early disease and might become an established treatment option for suitable patients. Further improvements in LT and postoperative management along with novel chemotherapeutic and biological agents may further improve the current outcomes. Larger, high quality studies are needed in each group of tumours.

FOOTNOTES

Author contributions: Mavroeidis VK conceptualised and designed the study; Borakati A and Mavroeidis VK did the literature search; Borakati A and Froghi F did the analysis, interpretation of data and drafted the original manuscript; Bhogal RH and Mavroeidis VK supervised and made critical revisions; All authors prepared the final draft and approved the final version.

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MINIREVIEWS

Research progress on the mitochondrial mechanism of age-related non-alcoholic fatty liver

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide. Reduced activity and slower metabolism in the elderly affect the balance of lipid metabolism in the liver leading to the accumulation of lipids. This affects the mitochondrial respiratory chain and the efficiency of β-oxidation and induces the overproduction of reactive oxygen species. In addition, the dynamic balance of the mitochondria is disrupted during the ageing process, which inhibits its phagocytic function and further aggravates liver injury, leading to a higher incidence of NAFLD in the elderly population. The present study reviewed the manifestations, role and mechanism of mitochondrial dysfunction in the progression of NAFLD in the elderly. Based on the understanding of mitochondrial dysfunction and abnormal lipid metabolism, this study discusses the treatment strategies and the potential therapeutic targets for NAFLD, including lipid accumulation, antioxidation, mitophagy and liver-protecting drugs. The purpose is to provide new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

Key Words: Non-alcoholic fatty liver disease; aging; Lipid metabolism; Mitochondria; Autophagy; Reactive oxygen species

Core Tip: The elderly are prone to a series of pathological damages such as liver fibrosis due to the decline of liver regeneration ability and immune response dysfunction. In addition, liver metabolism imbalance and mitochondrial dysregulation play a key role in the development of non-alcoholic fatty liver disease (NAFLD). And a treatment strategy for NAFLD was proposed in terms of abnormal lipid metabolism, mitophagy, and anti-oxidation, which provided new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

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INTRODUCTION

In the past several decades, ageing has become a research hot spot with the understanding of cancer and metabolic disorder-related diseases. The ageing liver is also becoming a public health problem[1]. Due to the decline in the regenerative ability of the liver and dysfunctions in the immune response, older people are more likely to suffer from non-alcoholic fatty liver disease (NAFLD), acute and chronic liver injury, liver fibrosis and other diseases. Studies have reported that the prevalence of NAFLD increases in the elderly, with a prevalence of < 30% in people under 40 years of age and > 50% in people over 60 years of age[2].

Currently, it is believed that the mechanism of development of NAFLD includes increased production of fat, increased dietary free fatty acid (FFA) levels, β-oxidative damage and dysfunction in very low density lipoprotein synthesis[3]. However, reduced activity and changes in diet structure lead to a continuous increase in body fat in the elderly. These factors lead to the accumulation of TGs in the liver and eventually cause age-related NAFLD[4]. Studies have reported that the accumulation of TG droplets in hepatocytes is not a harmful process in itself. On the contrary, it is considered an adaptive response to excessive lipid uptake or the production of fat[5], and this imbalance in TG synthesis and breakdown causes fatty degeneration of the liver[6]. In addition, the structural and functional changes in the mitochondria have been shown to be related to the pathogenesis of NAFLD. Ultramicroscopic analyses have demonstrated a disordered morphology of hepatocyte mitochondria in elderly patients with NAFLD, and the damage to the structure and function led to fatty degeneration of the liver and other injuries[3]. The changes in mitosis and fusion of mitochondria during ageing lead to the inhibition of mitochondrial phagocytosis[7]. Cell function can be affected further if the damaged mitochondria are not cleared in time[8].

Age-related diseases are the major challenge in the 21st century. Delaying ageing is a significant challenge that people want to overcome. Ageing has become a very serious risk factor. Ageing starts with molecular damage and eventually leads to the dysfunction of cells, tissues and organs[9]. Clinically, the core of biological ageing is the increased vulnerability to death. The structural and functional changes in the mitochondria have been proven to be related to the pathogenesis of NAFLD, the loss of mitochondrial DNA (mtDNA) in hepatocytes affects function, leading to hepatic steatosis and other injuries[10]. The present study reviewed the manifestations, role and mechanism of mitochondrial dysfunction in the progression of NAFLD in the elderly. In addition, the study discusses the treatment strategies for NAFLD based on the understanding of mitochondrial dysfunction and abnormal lipid metabolism to provide new ideas for the development of innovative drugs for the prevention and treatment of NAFLD.

AGE-RELATED NAFLD: THE ROLE OF LIPID METABOLISM

Lipid metabolism is an important and complex biochemical process in the human body. The liver has a strong ability to synthesize fat, but it cannot store fat. Fatty degeneration of the liver occurs when fat is not transported in time. Simultaneously, the body decomposes fat through the action of various enzymes to maintain the dynamic balance of lipid metabolism. An imbalance in lipid metabolism ensues when there is a problem in decomposition. Therefore, in the event of fatty liver-related diseases, the primary consideration is to identify dysfunctions in the lipid metabolism pathway[11].

Enzyme regulation of β-oxidation of fatty acids

Mitochondria ensure a continuous supply of energy and metabolites to the organism through the aerobic oxidation of fatty acids, and the transfer of fatty acids into the mitochondria for β-oxidation

needs to be regulated[12]. In the mitochondria, the rate of lipid oxidation is significantly related to the entry of long-chain fatty acids into the matrix as well as β-oxidation. The long-chain acyl-CoA synthetase catalyses the fatty acids to synthetize the acyl-coenzyme A. Considering that the inner mitochondrial membrane (IMM) is impermeable to coenzyme A, the exchange of coenzyme A and carnitine is essential. The enzyme carnitine palmitoyltransferase-1 (CPT-1) is located on the outer mitochondrial membrane (OMM) and catalyses the reversible transfer of acyl-coenzyme A groups (with a chain length of C12 to C18) to L-carnitine to form acylcarnitine esters[13]. This newly generated acylcarnitine is subsequently transferred into the mitochondrial matrix in exchange for free carnitine. This translocation step is mediated by carnitine-acylcarnitine translocase (gene name: *SLC25A20*). Once in the mitochondrial matrix, acyl groups are transferred back to coenzyme A *via* CPT2. Human CPT1 exists in three forms: Liver subtype (CPT1A), muscle subtype (CPT1B)[14] and cerebral subtype (CPT1C)[15]. CPT1A and CPT1B have similar functions but are expressed in different tissues. The deficiency of CPT1A is associated with increased levels of L-carnitine in plasma, which provides a clue for the diagnosis of CPT1A deficiency. At the cellular level, the lack of CPT1A activity in the liver leads to the inability to generate acylcarnitines that enter the mitochondria for oxidation. Therefore, CPT1 has become an important target in the regulation of mammalian lipid metabolism.

Abnormal lipid metabolism in the early stage of NAFLD induces oxidative stress, and mitochondrial dysfunction is also an important aspect of oxidative stress. Damage to the mtDNA is the main cause of mitochondrial structural damage. Gene damage occurs due to a direct attack on mtDNA by various lipid peroxidation products, resulting in the reduction of the synthesis of its encoded mitochondrial complex and ATP synthase. Gene damage directly affects mitochondrial function. Lipotoxicity-induced oxidative stress disorder reflects the close relationship between abnormal lipid metabolism and mitochondrial dysfunction (Figure 1).

Regulation of nuclear receptor peroxisome proliferator-activated receptor for β-oxidation of fatty acids

In addition to various enzymes, nuclear receptors can also affect the β-oxidation of fatty acids[16]. Steatosis is one of the pathological characteristics of NAFLD, and fat accumulation leads to the activation of the nuclear receptor, peroxisome proliferator-activated receptors (PPARs)[17]. Studies conducted in 1990 revealed that drug experiments could cause the proliferation of liver peroxisomes, through which PPAR was discovered and later designated as PPARα (NR1C1). Subsequently, two additional PPARs were identified, namely, PPARβ/δ (NR1C2) and PPARγ (NR1C3). The expression organs and functions of the three PPARs are very different, PPARα is mainly expressed in the liver and brown adipose tissue[18]; PPARβ/δ is widely expressed in vivo, with relatively high expression in brain, stomach and intestine[19]; PPARγ expression is highest in white and brown adipose tissue[20]. PPARα is highly expressed in the liver, which is the main regulatory protein involved in hepatic β-oxidation, and is considered to be an important target for regulating dyslipidemia. Studies have shown that mitochondrial β-oxidation is significantly reduced in the liver of PPARα deficient mice[16]. Fatty liver can be improved by the activation of PPARα and the enhancement of hepatic fatty acid β-oxidation. Studies that investigated liver tissues from patients with NAFLD and non-alcoholic steatohepatitis (NASH)[21] reported that the expression of PPARα decreased with the progression of fibrosis. PPARα stimulants lead to decreased expression of substances associated with hepatic steatosis by enhancing mitochondrial β-oxidation. PPARα is involved in mitochondrial β-oxidation, with CPT1 as the key enzyme, allowing fatty acids to reach the mitochondrial matrix through the IMM and subsequently be metabolised. Abnormal PPARα expression can increase the levels of fatty acids and store them in the liver as triglycerides. PPARβ has been reported infrequently and has multiple functions and distributions, mainly being involved in wound healing. PPARγ is an important transcription factor for cell differentiation.

Regulation of the breakdown of lipid droplets

Lipid droplets (LDs) have a unique structure and exist in almost all cells. They are specialised organelles for storing lipids in cells. LDs provide energy for cells, aid in biofilm synthesis[22], and prevent FFA-induced lipotoxicity and its influx into toxic lipid species[23]. The accumulation of LDs in non-adipose tissue is a pathological feature of metabolic diseases such as obesity, diabetes and atherosclerosis[24]. In most cases, LDs exist in the form of an emulsion, which is the dispersed phase of water in oil in an aqueous solution. When large amounts of LDs are present, cells synthesize neutral lipids, which then form droplets dispersed in the aqueous phase[25]. The mechanism of the biogenesis of LDs remains unclear. The mechanisms underlying LDs biogenesis remain unknown, but studies suggest that LDs are simple structures surrounded by endoplasmic reticulum (ER)-derived phospholipid monolayers with dynamically changing proteome modifications on the surface[26]. A group of proteins, including seipin or lipid storage-inducing transmembrane proteins, are thought to be important for the formation of LDs. According to recent research, LDs are created de novo by neutral lipid deposition between ER leaflets and directed LD germination from the ER's outer leaflet into the cytoplasm[27].

LDs play a core role in cellular metabolism and homeostasis. Along with enhanced synthesis, decreased catabolism of LDs is another potential source of hepatic steatosis. There are two main

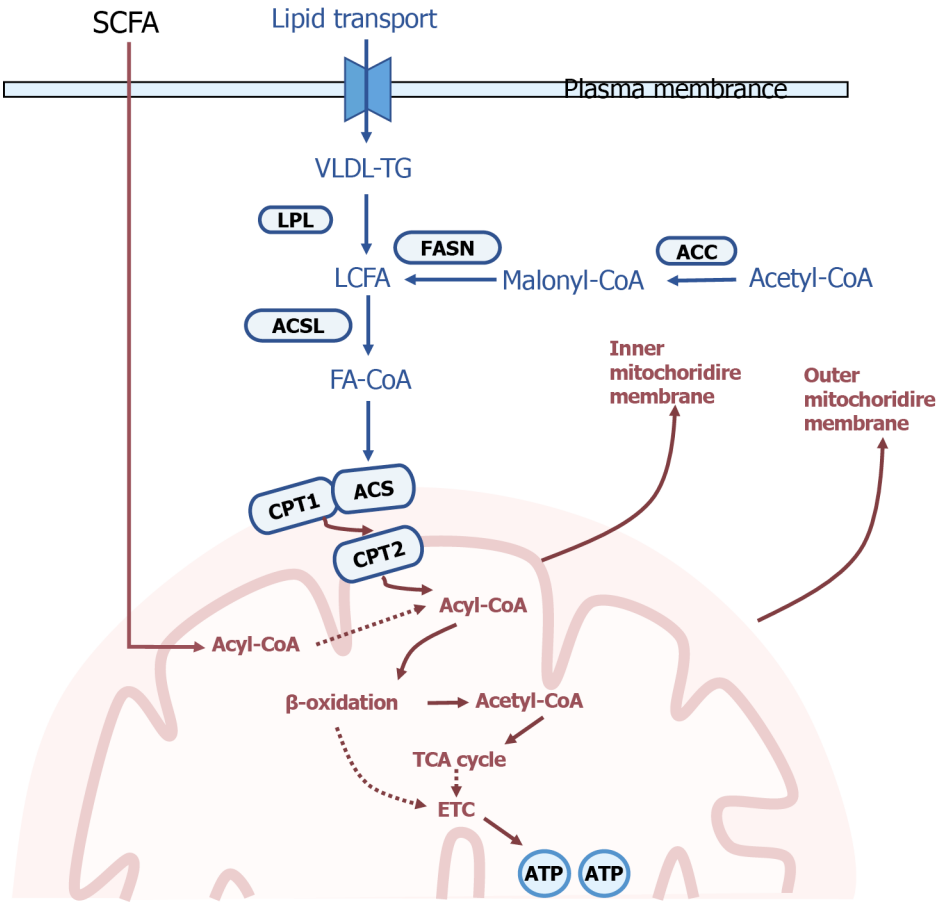

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Figure 1 Enzymatic regulation of fatty acid aerobic oxidation. SCFA: Short-chain-fatty acids; CPT1: Carnitine palmitoyltransferase-1; CPT2: Carnitine palmitoyltransferase-2; LCFA: Long-chain fatty acids; VLDL: Very low density lipoprotein; ACSL: Long-chain acyl-CoA synthetase; TCA: Tricarboxylic acid; ACS: Acyl-CoA synthetase; ACC: Acetyl-CoA carboxylase; ETC: Electronic respiratory chain; FASN: Fatty acid synthase.

pathways for the turnover of LDs in hepatocytes: conventional lipolysis and autophagy[28]. Lipolysis involves three key lipases, namely, hormone-sensitive lipase, adipose triglyceride lipase and monoacylglycerol lipase, which catalyse the rapid lipolysis of adipose tissue. In addition, some enzymes can directly interact with LDs to remove FAs from the triglyceride backbone stored in LDs one at a time. Furthermore, proteins also play an important role in the liver because the regulation of enzymes requires the participation of numerous hormones and growth factors through a variety of signal transduction pathways[29]. In addition to the above soluble lipases, hepatocytes also utilise the autophagy pathway to decompose LDs. Autophagy is mediated by lysosomes, and the formation of a special double-layer membrane vesicular structure during the process is called an autophagosome. In recent years, researchers have progressed in identifying the mechanism of other organelle-selective autophagy processes, such as mitochondrial phagocytosis and peroxidase phagocytosis[30]. Studies have found that lipid phagocytosis can be both non-selective and selective. Selective autophagy refers to the selective recognition and degradation of lipids, regulation of hepatocyte fat metabolism, and maintenance of intracellular lipid homeostasis.

Lipid metabolism in age-related NAFLD

Under normal circumstances, triglycerides are transported into the liver for lipid metabolism and are absorbed by hepatocytes. The main causes of NAFLD during ageing are an imbalance in hepatic metabolism, obesity, malnutrition and insulin resistance. However, there are few reports on the mechanism of development of age-related NAFLD, which needs further research in the future.

Obesity and metabolic abnormalities can be observed in elderly patients. The fat content in the body continues to increase with advancing age. Most of the fat exists in the form of triglycerides. The colour of the liver is closely related to the triglyceride content[31]. In addition to obesity, a low-quality diet and reduced activity in older patients can lead to malnutrition and muscle loss. Moreover, a low-quality diet can lead to an insufficient caloric supply and low protein levels, leading to an increase in the serum levels of FFAs. Furthermore, severe insulin resistance in skeletal muscles during ageing can induce the upregulation of the cholesterol regulatory element-binding protein 1, which inhibits β-oxidation and leads to fat deposition in the liver. Molecular substances related to NAFLD interfere with the cascade

reaction of the insulin signalling pathway and aggravate insulin resistance. Studies have also reported that compared with young individuals, glycogen synthesis in the muscles of elderly individuals decreases by 45%, the liver fat re-synthesis rate is twice higher, and the fasting blood triacylglycerol level is significantly higher, which increases by approximately three times after food[32].

ROLE OF MITOCHONDRIAL DYSREGULATION IN AGE-RELATED NAFLD

Mitochondria, which account for about one-fifth of the cell volume, are the organelles that mainly produce ATP in eukaryotic cells[33]. Almost all energy in the human body is generated by the tricarboxylic acid cycle and the electron transport chain of mitochondria, which is produced in the form of ATP. Mitochondria have inner and outer bilayers. The permeability of the inner membrane is lower than that of the outer membrane. The enzymes involved in the electron transport chain and ATP generation process are located in the inner membrane. An important feature of human ageing is a decrease in mitochondrial function in various tissues. Therefore, mitochondrial defects or dysregulation play a key role in ageing as well as diseases such as cancer[34].

Mitochondrial homeostasis

Mitochondria are dynamic organelles that continuously undergo fusion/division, and their homeostatic balance is essential for the maintenance of function. Many genes associated with mitochondrial homeostasis are human disease genes. Mitochondrial dynamics are regulated by two opposite processes, fusion and fission. The processes are essential to regulate mitochondrial morphology, number, function and sub-cellular distribution, as well as to maintain mitochondrial homeostasis to cope with stress[35]. Mitochondrial dynamics vary according to the developmental stage, age, cell type, environmental factors and genetic background. Mitochondrial homeostasis is affected during ageing. The structure of the mitochondria becomes abnormal with advancing age, biogenesis is reduced, and mtDNA mutation is increased[36].

During ageing, the changes in mitochondrial fission and fusion lead to the inhibition of mitochondrial phagocytosis, and further damage to cellular function occurs if the damaged mitochondria are not cleared in time. The process of mitochondrial fusion requires the inner and outer membrane proteins, optic atrophy 1 (OPA1) protein and mitotic fusion proteins (MFN1 and MFN2)[37]. Deletion of the mitotic fusion proteins blocks the fusion of the inner and outer membrane proteins (IMM and OMM), while deletion of OPA1 blocks the fusion of the IMM but not the OMM[38]. The genetic disappearance of OPA1 changes the morphology and activity of lysosomes, thus inducing the accumulation of autophagic substrates. Mitochondrial fission is mediated by several proteins, with mitochondrial motility-related proteins being central. When mitochondria divide, dynamin-related protein 1 (DRP1) is located in the proteins and factors on the OMM to form a ring structure. The ring is divided into two mitochondrial cristae through the GTP enzyme activity of DRP1. After cell division, DRP1 returns to the cytoplasm to participate in the next mitochondrial division[39].

Mitochondria maintain their morphology and function in cells through continuous fusion and division[40]. Studies have found that the mitochondria in the liver of mice with NAFLD become smaller and fragmented[41]. Steatosis and inflammatory reaction in the liver are aggravated in mice with a knockdown of the MFN2 gene that controls mitochondrial fusion, promoting the progression of liver fibrosis and liver cancer. Wang *et al*[42] reported that mice with NAFLD and hepatic mitochondrial DRP1 gene knockout had mitochondrial fission disorder, which directly promoted ER stress and hepatocyte death. In the pathological state of age-related NAFLD, oxidative stress in the liver aggravates mitochondrial functional and structural damage. The damage can manifest as an imbalance in the regulation of mitochondrial quality, such as the splitting of mitochondria, increased autophagy and fusion, and reduced biosynthesis, thereby affecting liver function and energy metabolism and promoting NAFLD[43,44].

Mitochondrial reactive oxygen species

The acellular production of reactive oxygen species (ROS) is an inevitable process. Under normal circumstances, oxygen metabolism generates an adequate amount of ROS required for detoxification. An excessive amount of toxins will lead to tissue damage and inflammation; however, cells have several defense systems to fight them. Most data indicate that oxidative damage increases in older adults. Some studies have reported that the antioxidant defense capacity decreases with age, while others have not reported any changes[45]. Compared with other rodents, naked mole rats, a long-lived rodent, have an increased amount of ROS. However, these rats also have an increased free radical scavenging ability, which does not decline with age[46]. Intracellular ROS may also play important roles in intracellular signaling, which may be beneficial to the ageing process. Therefore, in our opinion, ROS are not necessarily harmful, and the balance between ROS and scavenging may be the key.

It has been found that the mitochondrial respiratory chain and β-oxidation are gradually impaired in hepatocytes of NAFLD, which induces the generation of excess amounts of ROS[47]. Superoxide anions, hydroxyl radicals, peroxy radicals, and other non-radicals that can produce free radicals are all

members of the family of free radicals known as ROS[48]. The overproduction of ROS is related to oxidative damage to lipids, DNA and proteins[49,50]. According to the multiple parallel hit theory, oxidative stress is considered a major factor in liver injury and disease progression[51]. Oxidative stress has increasingly become one of the important pathological causes of the development of NAFLD, and it is the link between simple steatosis and symptoms of NASH[52].

Lipids are one of the main sources of mitochondrial ROS in NAFLD. Mitochondria can produce ROS during oxidative phosphorylation (OXPHOS), which can trigger mitochondrial dysfunction (such as DNA, proteins, lipids and other molecules) by interacting with mitochondria and cellular components [53]. Mitochondrial function and ROS production are considered important regulators of lifespan. Mitochondrial dysfunction leads to the overproduction of ROS. Oxidative damage may be involved in various pathological processes. At present, an imbalance in the mitochondrial redox state is considered the main cause of cell damage. The interaction between mitochondria and peroxisomes regulates metabolic and redox signaling pathways through the mitochondrial delivery system[54]. Mitochondrial ROS are considered an important factor in the development of liver disease. The production of mitochondrial ROS may lead to fatal cell damage due to the impairment of several bioenergetic responses involved in OXPHOS[55]. In addition, due to its proximity to ETC, mtDNA is extremely prone to oxidative damage, leading to DNA breakage and mutation[56].

Mitophagy

The understanding of the physiological role of autophagy in mammals has increased in the past decade. Autophagy is associated with many physiological processes[57]. The main function of autophagy is to degrade endogenous biomacromolecules and recycle cellular substances. There are three main forms of autophagy[58], namely, microautophagy, macroautophagy and chaperone-mediated autophagy. The difference lies in the mode of transportation. Autophagy has become a potential anti-ageing mechanism [59]. Autophagy declines with age and the expression of several key indicators in the autophagy pathway (such as ATG5 and ATG7) show a downward trend in the brain of older people[60]. Caloric restriction and exercise can enable autophagy to delay aging-associated degeneration[61].

The ageing of cellular mitochondria gradually makes it inefficient and potentially toxic. An acute injury can increase the permeability of the mitochondrial membrane, thus triggering apoptosis or necrosis. Autophagy inhibits inflammation through phagocytosis of dysfunctional or damaged mitochondria, preventing unnecessary cell loss[62]. Autophagy can not only regulate lipid metabolism and insulin resistance[63] but also degenerative diseases caused by the reduced expression of autophagy or mitophagy genes, including inflammation and cell population death due to lack of quality control. A growing body of evidence also shows that hepatic autophagy is impaired in NAFLD[64], thus confirming the theory that the combination of mitochondrial dysfunction and insufficient autophagy may lead to a variety of age-related diseases, and making autophagic flux a potential pharmacological target in NAFLD.

Presently, two main mechanisms are known to regulate mitochondrial autophagy, namely, mitophagy regulated by parkin/PINK1[65] and receptor-regulated autophagy[66]. Parkin is an E3 ubiquitin ligase located in the downstream. PINK1 is a serine/threonine kinase located in the upstream. Autophosphorylation of PINK1 is essential for ubiquitin recognition and can promote the translocation of phosphorylation for parkin from the cytosol to mitochondria. In the event of mitochondrial damage, the membrane potential decreases, leading to the accumulation of PINK1 in the OMM, attracting parkin to attach to mitochondria and causing further autophagy. In addition, Nip3-like protein X (NIX) and Bcl-219 kDa interacting protein (BNIP3) are outer membrane proteins associated with autophagy and apoptosis under hypoxia, which can act as mitochondrial autophagy receptors. Studies have confirmed that NIX can also participate in the process of parkin-dependent mitochondrial autophagy. It can be ubiquitinated as a substrate of parkin and recruit the LC3 adaptor protein NBR1 to target mitochondria to autophagosomes and induce mitochondrial autophagy (Figure 2)[67].

Mitochondrial dysfunction is characterised by an imbalance in mitochondrial homeostasis, ROS overproduction, autophagy abnormalities, respiratory chain disorders and mtDNA damage. A large number of studies have demonstrated structural and functional alterations in the mitochondria of hepatocytes in NAFLD (Figure 3)[68-70].

Mitochondria and gut microbiome

Appreciation of the role played by the gut microbiome has increased rapidly in recent years[71-73]. Comprehensive studies on the aetiology of NAFLD induced by excess nutrition and obesity have shown that changes in the gut microbiome are crucial in the development of NAFLD[74]. Dysregulation of gut flora has been shown to be an important factor in age-related pathology.

Mitochondria regulate intestinal function, including barrier defense of the intestinal epithelial cells [75-77]. Studies have shown that mitochondrial dysfunction in colonic epithelial cells induced by the use of dinitrophenol leads to dysfunction of the intestinal barrier[78], suggesting that mitochondrial dysfunction impairs the functional integrity of intestinal epithelial cells. MitoTEMPO, an antioxidant that targets mitochondria, inhibits these barrier defects, suggesting that mitochondrial stability is important for maintaining intestinal barrier function[71]. Unbalanced gut flora may contribute to the development and progression of multiple diseases. Recently, disorders of the intestinal flora have been

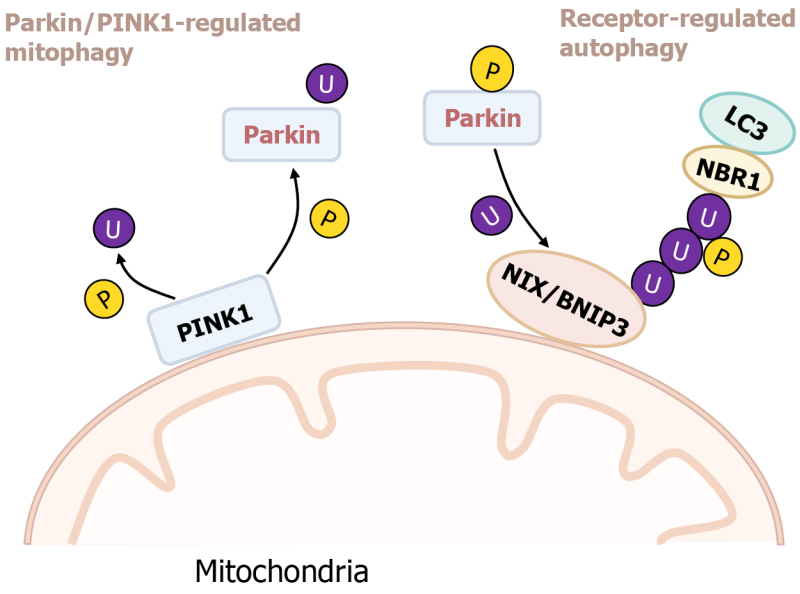

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Figure 2 Mitochondrial depolarization activates the PINK1/Parkin pathway to mediate mitophagy. NIX: Nip3-like protein X; BNIP3: Bcl-219 kDa interacting protein.

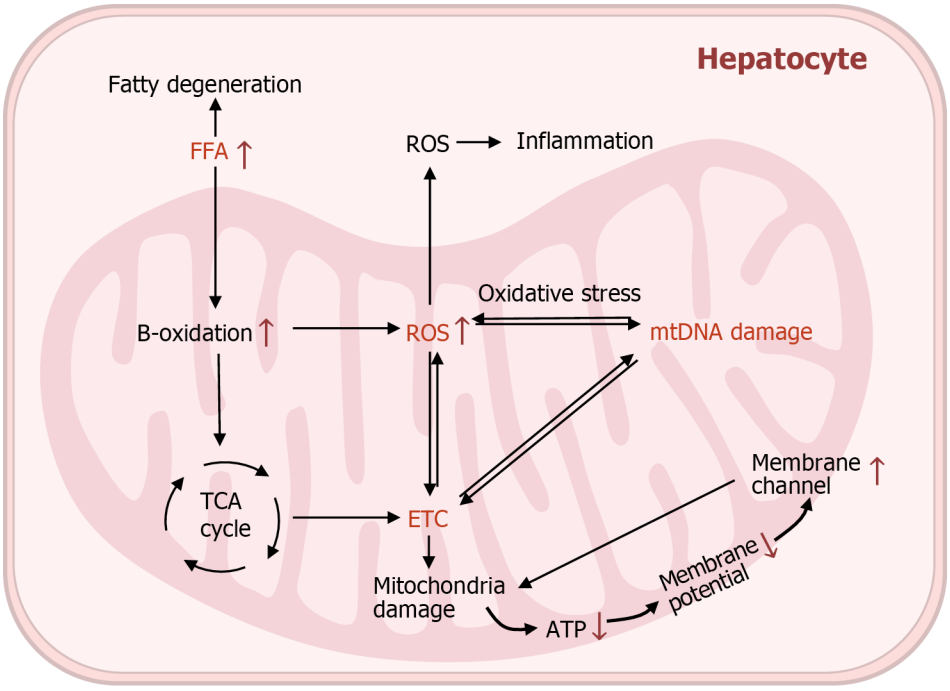

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Figure 3 Mitochondrial depolarization activates the PINK1/Parkin pathway to mediate mitophagy. FFA: Free fatty acid; ROS: Reactive oxygen species; TCA: Tricarboxylic acid; mtDNA: Mitochondrial DNA; ETC: Electronic respiratory chain.

implicated in liver diseases, such as hepatitis, cirrhosis and NAFLD[74].

THERAPIES TARGETING MITOCHONDRIAL FUNCTION IN AGE-RELATED NAFLD

Currently, there is no approved drug treatment for NAFLD. Hannah *et al*[79] found that a 3%-5% weight loss in patients with NAFLD slowed the progression of the condition. Although lifestyle changes through appropriate diet and exercise have been shown to be beneficial, most patients find it difficult to achieve and maintain the same. Therefore, identifying effective therapeutic drugs is an active area of

research. Clinically, the pharmacological agents for NAFLD mainly include lipid-lowering drugs, liver-protecting drugs, antioxidants, autophagy inhibitors and insulin sensitizers.

PPAR ligand therapy

The disorder of fat metabolism in the human body is one of the important reasons for the development of NAFLD. Aiming at the accumulation of liver fat, the PPAR ligand can be considered a possible therapeutic agent for NAFLD. Regulating lipid metabolism and reducing blood lipids is also a popular approach for the treatment of NAFLD[80]. The latest evidence from preclinical and clinical studies confirms the potential of PPAR ligands to treat this series of liver diseases. For example, elafibranor, a PPAR agonist[81], can increase mitochondrial fatty acid oxidation and oxidative phosphorylation and reduce the flow of fatty acids from adipose tissue to the liver. Presently, a phase III clinical trial (NCT02704403) has been completed.

Hepatoprotective drugs

Hepatoprotective drugs are commonly used as adjuvant in the treatment of NAFLD, which can not only protect the liver but also resist oxidation, inflammation and fibrosis and prevent the probability of malignant transformation of liver disease. Common hepatoprotective drugs include polyene phosphatidylcholine and obeticholic acid (OCA). Polyene phosphatidylcholine has anti-oxidative and anti-inflammatory effects that aid in reducing liver cell damage and even apoptosis and can effectively target the pathological symptoms caused by NAFLD. The effect of its combination with metformin in the treatment of patients with NAFLD and NASH is significantly better than that of monotherapy[82]. OCA is a farnesoid X receptor agonist. As a synthetic lipophilic bile acid, OCA can effectively reduce hepatic steatosis in mice[83].

Antioxidant drugs for inhibiting hepatocyte apoptosis

Vitamin E is an antioxidant and has been proven one of the more effective drugs for the treatment of NAFLD. Clinical studies have reported significant improvement in blood lipid biochemical indicators and histopathological changes in the liver after oral administration of VE at 800 IU/d for 96 wk. The results indicated that VE was beneficial for the treatment of NAFLD and NASH[84]. Sanyal *et al*[85] found that oral VE at 800 IU/d for 2 years could improve hepatic steatosis and inflammatory injury. Lv *et al*[86] hypothesised that the accumulation of ROS was the process of quiescent cells entering the cell cycle. Through experiments, they confirmed that the expression of cell cycle regulators P16 and P38 was related to the production of ROS, which provided a feasible therapeutic target for scavenging ROS and improving NAFLD.

Improve mitophagy

Improving mitophagy has been a hot topic in the treatment of NAFLD in recent years. Wu *et al*[87] found that a high-fat diet induced an increase in the expression of a mixed series of protein kinase-like domains (MLKL). MLKL can combine with liver mitochondria to stimulate the production of ROS and transfer it to the autophagosome membrane to inhibit the autophagy of injured cells and exacerbate liver injury. Therefore, MLKL-knockout models can improve the autophagy defect of injured hepatocytes and alleviate the progression of NAFLD. Studies have suggested the feasibility of inducing autophagy in the treatment of NAFLD and the application of related targets. However, excessive induction of autophagy may aggravate liver injury; therefore, maintaining the balance between them is the key to treating NAFLD.

CONCLUSION

In summary, as the central link of energy metabolism, mitochondria play an important role in maintaining the normal structure and function of age-related NAFLD. There is a concomitant and progressive relationship in the process of age-related NAFLD, from fatty degeneration to a series of pathological lesions, such as inflammation and liver fibrosis. However, whether a mitochondrial disorder of hepatocytes is the cause of the progression of age-related NAFLD or the result of abnormal lipid metabolism and lipotoxicity cannot be fully confirmed[88]. The occurrence of mitochondrial dysfunction is closely related to the disorders of dynamic balance, mtDNA deletion, excessive production of ROS, abnormal mitophagy and abnormal fatty acid oxidation. A better understanding of the mechanism of mitochondrial dysfunction will be helpful to develop new therapeutic strategies for NAFLD. In the next few years, the availability of therapeutic options for NAFLD is expected to curb the rising trend of related diseases. More rigorous experiments should be designed to validate the results and develop more targeted drugs that regulate mitochondrial function to treat NAFLD, thus achieving the transformation from basic theory to clinical practice.

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ORIGINAL ARTICLE

Case Control Study

Gut microbiota predicts the diagnosis of celiac disease in Saudi children

Mohammad El Mouzan, Asaad Assiri, Ahmed Al Sarkhy

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Abstract

BACKGROUND

Celiac disease (CeD) is a multisystem immune-mediated multifactorial condition strongly associated with the intestinal microbiota.

AIM

To evaluate the predictive power of the gut microbiota in the diagnosis of CeD and to search for important taxa that may help to distinguish CeD patients from controls.

METHODS

Microbial DNA from bacteria, viruses, and fungi, was isolated from mucosal and fecal samples of 40 children with CeD and 39 controls. All samples were sequenced using the HiSeq platform, the data were analyzed, and abundance and diversities were assessed. For this analysis, the predictive power of the microbiota was evaluated by calculating the area under the curve (AUC) using data for the entire microbiome. The Kruskal-Wallis test was used to evaluate the significance of the difference between AUCs. The Boruta logarithm, a wrapper built around the random forest classification algorithm, was used to identify important bacterial biomarkers for CeD.

RESULTS

In fecal samples, AUCs for bacterial, viral, and fungal microbiota were 52%, 58%, and 67.7% respectively, suggesting weak performance in predicting CeD. However, the combination of fecal bacteria and viruses showed a higher AUC of 81.8 %, indicating stronger predictive power in the diagnosis of CeD. In mucosal samples, AUCs for bacterial, viral, and fungal microbiota were 81.2%, 58.6%, and 35%, respectively, indicating that mucosal bacteria alone had the highest predictive power. Two bacteria, *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, in fecal samples and one virus, *Human_endogenous _retrovirus_K*, in mucosal samples are predicted to be "important" biomarkers, differentiating

celiac from nonceliac disease groups. *Bacteroides intestinalis* is known to degrade complex arabinoxylans and xylan which have a protective role in the intestinal mucosa. Similarly, several *Burkholderiales* species have been reported to produce peptidases that hydrolyze gluten peptides, with the potential to reduce the gluten content of food. Finally, a role for *Human_endogenous _retrovirus_K* in immune-mediated disease such as CeD has been reported.

CONCLUSION

The excellent predictive power of the combination of the fecal bacterial and viral microbiota with mucosal bacteria alone indicates a potential role in the diagnosis of difficult cases of CeD. *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, which were found to be deficient in CeD, have a potential protective role in the development of prophylactic modalities. Further studies on the role of the microbiota in general and *Human_endogenous _retrovirus_K* in particular are needed.

Key Words: Celiac disease; Microbial signature; Children; Saudi Arabia

Core Tip: Celiac disease (CeD) is known to be associated with the microbiota. In this study, the combination of bacterial and viral taxa in stools and mucosal bacterial taxa were the strongest predictors of celiac disease. In addition, we report important bacterial markers, namely, *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*, which were reduced in children with CeD, suggesting a protective role in children with CeD.

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INTRODUCTION

Celiac disease (CeD) is an immune-mediated condition with multisystem clinical expression[1,2]. The disease is distributed worldwide, and the incidence is increasing. The global seroprevalence and biopsy-proven prevalence are estimated to be 1.4% and 0.7%, respectively[3]. In the Kingdom of Saudi Arabia (KSA), a seroprevalence between 1.5% and 3% and a biopsy-proven prevalence of 1% are some of the highest observed rates in the world[4,5]. The pathogenesis of CeD is multifactorial, requiring genetic susceptibility in the form of human leukocyte antigen DQ2 and DQ8 genotypes and exposure to gluten-containing food[6]. In the KSA, high prevalence of genetic susceptibility of 47% has been reported[7]. Although genetic susceptibility and exposure to gluten-containing food are necessary, not all genetically susceptible individuals develop CeD. Moreover, in some cases, CeD develops later in life after many years of gluten ingestion, indicating that other factors may be important in loss of tolerance to gluten and development of clinical disease[8,9]. Microbial dysbiosis associated with CeD is thought to be one of the important environmental factors contributing to loss of tolerance to gluten and thereby playing a role in pathogenesis of CeD[10,11]. However, identification of microbial markers and the predictive power of microbial dysbiosis in CeD have rarely been reported[12]. Therefore, the objectives of this study were to determine the predictive power of the gut microbial community in the diagnosis of CeD and to search for taxa that may be important in differentiating children with CeD from those without CeD.

MATERIALS AND METHODS

Shotgun metagenomic analysis of fecal and duodenal mucosal samples from children with new onset CeD was performed for bacteria, viruses, and fungi. Briefly, there were 40 children with CeD. Patients were eligible if they had confirmed CeD by standard criteria and not received antibiotics for at least 6 mo. They were enrolled as they presented to clinics. There were 39 non-CeD controls, including 20 school children who were clinically healthy who provided stool samples and 19 from whom tissue samples from the second part of the duodenum were collected during diagnostic endoscopy performed for clinical indications. The children with CeD and controls were enrolled in the study after consent/assent. All children were recruited from King Khaled University Hospital and King Fahad Medical City,

both institutions are in Riyadh (KSA). Microbial DNA was isolated and sequenced using the HiSeq platform. The results included abundance and diversity analyses for bacteria, viruses, and fungi that were recently reported[13-15].

For the purpose of this report, receiver operating characteristic (ROC) analysis with calculation of the area under the curve (AUC) was used to assess the predictive power of the gut microbiota in the diagnosis of CeD. ROC analysis and calculation of the AUC for discrimination using data regarding microbial communities, including bacteria, viruses, and fungi, in stool and mucosa were performed. Boruta analysis was used to identify important taxa that may differentiate children with CeD from non-CeD controls. In brief, the Boruta logarithm is a wrapper built around the random forest classification algorithm implemented in the R package random forest[16]. The Boruta process consists of assigning an 'importance' score to each variable and identifying a threshold above which the variables are deemed important and below which they are not. This process is repeated to establish reproducibility and robustness and therefore generates many 'importance' scores for each taxon. Species-level relative abundance data were used to generate shadow variables to predict taxa that may be important in distinguishing celiac from nonceliac groups[17]. Sensitivity and specificity were calculated based on the output from a random forest classifier[18]. Bioinformatics and statistical analyses were performed by specialists at Cosmos ID, United States (https://www.cosmosid.com/).

RESULTS

The areas under the curve for the bacterial, viral, and fungal microbiota in fecal samples were 52%, 58%, and 67.7%, respectively, suggesting poor performance for each, in predicting CeD. However, the combination of fecal bacteria and viruses revealed a higher AUC of 81.8 %, indicating a stronger predictive power for the diagnosis of CeD (Figure 1). Nevertheless, the difference between the AUC of the bacterial microbiome alone and the combined bacterial and viral microbiomes showed borderline significance (P = 0.05211).

The areas under the curve for the bacterial, viral, and fungal microbiota in mucosal samples were 81.2%, 58.6%, and 35%, respectively, indicating the the highest predictive power for mucosal bacteria alone (Figure 2). The difference in AUC between mucosal and fecal bacteria was significant (P = 0.01885).

The scores for the confirmed important variables are summarized in Table 1, including the mean, median, minimum, and maximum importance values. The 'Decision' column indicates 'Confirmed' for the microbiota above the threshold set by Boruta analysis. The microbiota including two bacteria (*Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*) and one virus (*Human_endogenous _retrovirus_K*), was confirmed to be important for distinguishing between CeD and non-CeD groups.

The results of the Boruta random forest algorithm analysis are illustrated in Figure 3. Two bacteria (*Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*) in fecal samples and one virus in mucosal samples (*Human_endogenous _retrovirus_K*) were predicted to be "important" for differentiating celiac from nonceliac disease groups. No fungal species were found to be important.

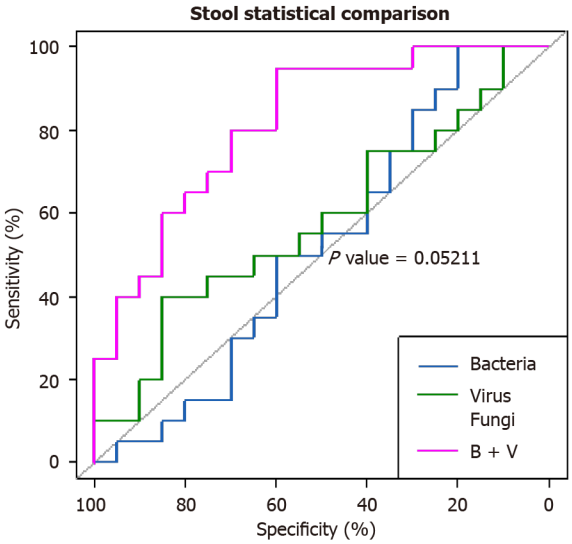
DISCUSSION

The role of the microbiota in predicting diseases in general has been largely reported. However, to our knowledge, the sensitivity and specificity of the gut microbiota in distinguishing CeD from non-CeD has not been reported thus far. In this study, the finding of a high AUC of the combination of bacteria and viruses in fecal samples (81.8%) indicates excellent predictive power with potential use in the diagnoses of difficult cases of CeD. Similarly, the significantly higher AUC for bacteria (81.2%) in the mucosal than in the fecal samples indicates stronger predictive power for mucosal bacteria (P = 0.01885). However, this was not surprising, as CeD is mainly a mucosal small bowel disease. These findings may have potential applications in the diagnosis of difficult cases of CeD.

Identification of the "important" specific microbiota in CeD in the form of *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* has not been previously reported. *Bacteroides intestinalis* belongs to the *Bacteroides* genus, members of which are known to degrade complex arabinoxylans and xylan from dietary fibers, including wheat, rye, oat, and barley[19]. These degradation products, including butyrate and ferulic acid, have been shown to have a protective role in the intestinal mucosa[20-22]. *Burkholderiales bacterium 1-1-47* is an unclassified bacterium belonging to the order *Burkholderiales*, class *Betaproteobacteria* and phylum *Proteobacteria*[23]. Several *Burkholderiales* species and *Burkholderia gladioli* in particular have been reported to produce peptidases that hydrolyze gluten peptides, with the potential to reduce the gluten content of food[24]. Accordingly, reports of decreased abundance of both *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in samples from children with CeD[13], indicate a potential protective role against the effects of gluten-containing grains.

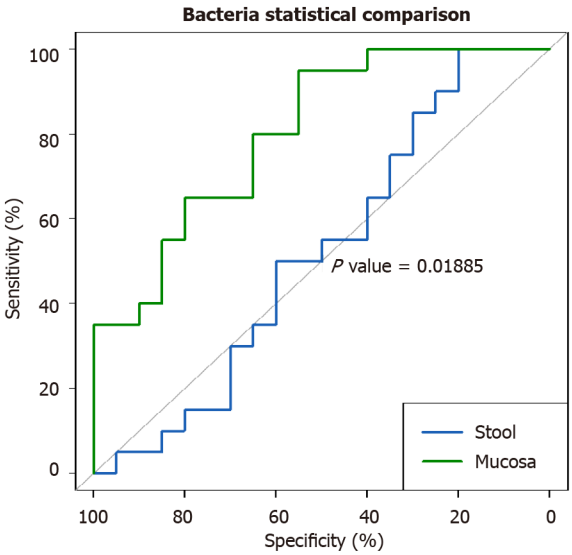
Table 1 Scores of important microbiota identified by Boruta analysis

Microbial species	Mean importance	Median importance	Minimum importance	Maximum importance	Decision
Bacteroides_intestinalis	6.92517709	7.70811062	1.25328634	9.93239532	Confirmed
Burkholderiales_bacterium_ 1_1_47	5.39952346	5.77233858	-1.307345	9.24744767	Confirmed
Human_endogenous _retrovirus_K	9.95761324	0.6340023	3.26946721	3.8621105	Confirmed



DOI: 10.3748/wjg.v29.i13.1994

Figure 1 Comparative area under the curves of the fecal microbiota show that the combination of bacteria and viruses was the strongest predictor of celiac disease. However, the difference between the area under the curve of bacteria alone and combined bacteria and viruses was borderline significant (*P* = 0.05211). B + V: Bacteria plus viruses.



DOI: 10.3748/wjg.v29.i13.1994

Figure 2 Comparison between mucosal and fecal bacterial area under the curves shows that mucosal bacteria were significantly stronger predictors of celiac disease (*P* = 0.01885).

Confirmation of *Human_endogenous _retrovirus_K* virus as important in differentiating CeD from non-CeD groups is interesting. This group of viruses has been suggested to have a role in immunity and autoimmune disorders. They can contribute to host protection or to damage, suggesting a subtle balance between the persistence of human endogenous retroviruses expression and maintenance of a basal immune alert[25]. Although a recent study found increased expression in children with CeD[26], identi-

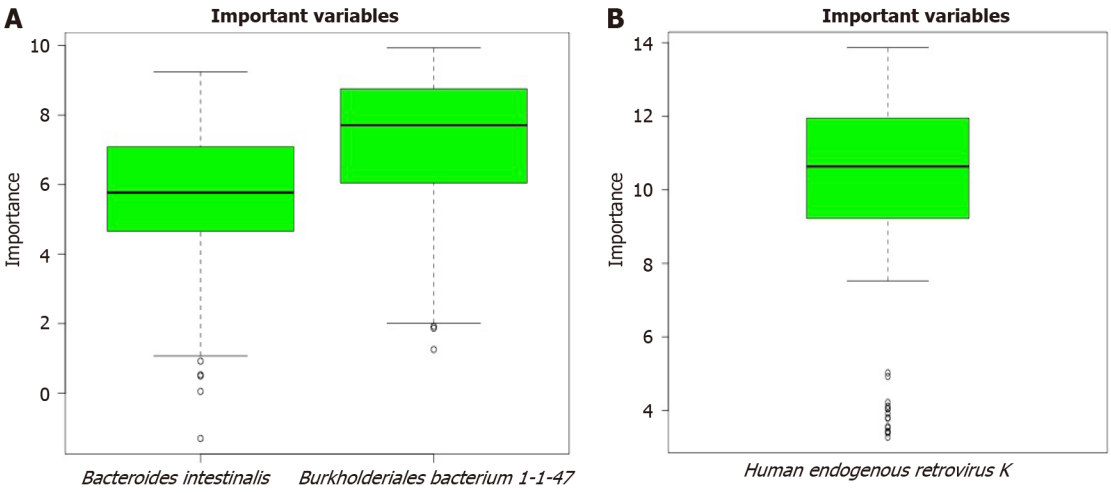


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Figure 3 The microbiota predicted important by Boruta random forest algorithm. These included two bacteria in fecal samples. A: *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47*; B: One virus mucosal samples, *Human_endogenous _retrovirus_K*.

fication of *Human_endogenous _retrovirus_K* as important and the significantly reduced abundance in children with CeD[13], should prompt further investigation of the role of these viruses in children with CeD.

Study limitation

This study had a relatively small sample size. In addition, the non-CeD controls were not completely healthy although they do not have CeD as TTG-A, endoscopy, and duodenal tissue histopathology were normal. However, the relatively small size might be partially compensated for by the use of the shotgun metagenomic analysis. Since this is the first report on microbiota accuracy and identification of important bacteria and viruses, but not fungi, further studies with larger sample sizes are needed.

CONCLUSION

The high AUCs of mucosal bacteria and the combination of fecal bacteria and viruses indicate a potential role in the diagnosis of difficult cases of CeD. In addition, identification of important bacteria as decreased abundances of *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in children with CeD, suggests a protective role with the potential for the development of preventive and adjuvant microbial therapy for CeD. The importance of *Human_endogenous _retrovirus_K* is interesting. However, further studies with larger sample sizes, are needed to improve our understanding of the role of the microbiota in CeD.

ARTICLE HIGHLIGHTS

Research background

Dysbiosis associated with celiac disease (CeD) is well known and beneficial and harmful associations have been reported.

Research motivation

The role of the microbiota in predicting CeD has rarely been described.

Research objectives

To search for a microbial signature that may help in the diagnosis and prevention of CeD.

Research methods

Metagenomic analysis of microbial DNA in mucosa and stool of children with newly diagnosed CeD calculation of the area under the curve to evaluate the predictive power of the whole microbiota and use of rendom forest analysis to identify important microbes in distinguishing CeD groups from controls.

Research results

Very high discriminatory power of combined bacteria and viruses (81.8%) in fecal samples and bacteria only in mucosal samples (81.2%). *Bacteroides intestinalis* and *Burkholderiales bacterium 1-1-47* in fecal samples were demmed important.

Research conclusions

The excellent predictive power of microbiota may help in the diagnosis of difficult cases of CeD. The identification of important specific bacterial species that are reduced in CeD may have a potential protective role.

Research perspectives

Future research in this area with larger sample sizes is needed to clarify the role of microbiota in the diagnosis and prevention of CeD.

FOOTNOTES

Author contributions: El Mouzan M designed the study, interpreted the data, drafted, and revised the manuscript; Assiri A and Al Sarkhy A participated equally in the data management and revision; and all authors approved the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Preoperative prediction of macrotrabecular-massive hepatocellular carcinoma through dynamic contrast-enhanced magnetic resonance imaging-based radiomics

Yang Zhang, Dong He, Jing Liu, Yu-Guo Wei, Lin-Lin Shi

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Abstract

BACKGROUND

Macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) is closely related to aggressive phenotype, gene mutation, carcinogenic pathway, and immunohistochemical markers and is a strong independent predictor of early recurrence and poor prognosis. With the development of imaging technology, successful applications of contrast-enhanced magnetic resonance imaging (MRI) have been reported in identifying the MTM-HCC subtype. Radiomics, as an objective and beneficial method for tumour evaluation, is used to convert medical images into high-throughput quantification features that greatly push the development of precision medicine.

AIM

To establish and verify a nomogram for preoperatively identifying MTM-HCC by comparing different machine learning algorithms.

METHODS

This retrospective study enrolled 232 (training set, 162; test set, 70) hepatocellular carcinoma patients from April 2018 to September 2021. A total of 3111 radiomics features were extracted from dynamic contrast-enhanced MRI, followed by dimension reduction of these features. Logistic regression (LR), K-nearest neighbour (KNN), Bayes, Tree, and support vector machine (SVM) algorithms

were used to select the best radiomics signature. We used the relative standard deviation (RSD) and bootstrap methods to quantify the stability of these five algorithms. The algorithm with the lowest RSD represented the best stability, and it was used to construct the best radiomics model. Multivariable logistic analysis was used to select the useful clinical and radiological features, and different predictive models were established. Finally, the predictive performances of the different models were assessed by evaluating the area under the curve (AUC).

RESULTS

The RSD values based on LR, KNN, Bayes, Tree, and SVM were 3.8%, 8.6%, 4.3%, 17.7%, and 17.4%, respectively. Therefore, the LR machine learning algorithm was selected to construct the best radiomics signature, which performed well with AUCs of 0.766 and 0.739 in the training and test sets, respectively. In the multivariable analysis, age [odds ratio (OR) = 0.956, P = 0.034], alpha-fetoprotein (OR = 10.066, P < 0.001), tumour size (OR = 3.316, P = 0.002), tumour-to-liver apparent diffusion coefficient (ADC) ratio (OR = 0.156, P = 0.037), and radiomics score (OR = 2.923, P < 0.001) were independent predictors of MTM-HCC. Among the different models, the predictive performances of the clinical-radiomics model and radiological-radiomics model were significantly improved compared to those of the clinical model (AUCs: 0.888 *vs* 0.836, P = 0.046) and radiological model (AUCs: 0.796 *vs* 0.688, P = 0.012), respectively, in the training set, highlighting the improved predictive performance of radiomics. The nomogram performed best, with AUCs of 0.896 and 0.805 in the training and test sets, respectively.

CONCLUSION

The nomogram containing radiomics, age, alpha-fetoprotein, tumour size, and tumour-to-liver ADC ratio revealed excellent predictive ability in preoperatively identifying the MTM-HCC subtype.

Key Words: Hepatocellular carcinoma; Macrotrabecular-massive subtype; Algorithms; Radiomics; Models; Nomogram

Core Tip: Radiomics features can be used to predict the macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) subtype. The logistic regression algorithm can improve the accuracy and stability of predicting MTM-HCC. Age, alpha-fetoprotein, tumour size, tumour-to-liver apparent diffusion coefficient ratio, and radiomics score were significant independent predictors of MTM-HCC. The nomogram based on radiomics, clinical and radiological features can serve as a noninvasive biomarker to preoperatively identify MTM-HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide, with a 5-year recurrence rate of 70% after surgical resection[1]. The poor prognosis of patients with HCC is closely related to histopathological subtypes[2]. Recently, a newly identified histopathological subtype was named "macrotrabecular-massive HCC (MTM-HCC)" and was officially included in the new classification of HCC by the World Health Organization in 2019[3]. As the most common subtype with metastatic potential[4], MTM-HCC is closely related to gene mutation, carcinogenic pathway, and immunohistochemical markers[5] and is a strong independent predictor of early recurrence and poor prognosis[6,7].

Early diagnosis and appropriate treatment of MTM-HCC are beneficial to prevent early recurrence and improve prognosis. Current studies have shown that radiofrequency ablation is not recommended for patients with aggressive HCC, while performing resection with wide margins or anatomical hepatectomy and shorter follow-up intervals may be recommended for monitoring[8,9]. MTM-HCC shows an aggressive phenotype[10]. Therefore, an accurate preoperative diagnosis of MTM-HCC can

provide the best individualized treatment plan.

With the development of imaging technology, successful applications of magnetic resonance imaging (MRI) have been reported in identifying the MTM-HCC subtype[11]. Several studies[11-14] have reported that MTM-HCC has characteristic MRI features, such as intratumor substantial necrosis and intratumor fat. However, these studies mainly focused on the qualitative analysis of imaging features. Radiomics, as an objective and beneficial method for tumour evaluation, is used to convert medical images into high-throughput quantification features that greatly push the development of precision medicine[15]. A recent study has also shown that radiomics is a superior tool for predicting MTM-HCC. However, this study only enrolled 88 patients and lacked a validation set, and there was limited reproducibility of the results. In addition, a highly accurate and reliable radiomics model can be constructed by comparing different machine learning algorithms. Therefore, we hypothesize that valuable MRI-based radiomics features could be extracted and a noninvasive and comprehensive model could be constructed through machine learning to better predict MTM-HCC.

In this study, we aimed to apply MRI-based radiomics to preoperatively predict MTM-HCC using different machine learning algorithms to build the best radiomics signature and to establish and validate the nomogram by combining preoperative clinical and radiological features to improve the decision-making process in clinical practice.

MATERIALS AND METHODS

Patients

This retrospective study was approved by our institutional review board, and the requirement for written informed consent was waived. Between April 2018 and September 2021, patients with suspected HCCs who underwent preoperative liver MRI examinations were included. The inclusion criteria included the following: (1) Pathologically proven primary HCC; (2) received liver MRI examinations one month before surgery; and (3) underwent curative hepatectomy. The exclusion criteria included: (1) Tumours less than 1 cm; (2) preoperative antitumour treatments; (3) poor image quality caused by metal or motion artifacts; and (4) lack of complete clinicopathological data. Finally, 232 patients were enrolled and divided into training (n = 162) and test (n = 70) sets at a ratio of 7:3. The patient recruitment process is shown in Figure 1.

MRI examinations

All MRI examinations were performed on a 3.0 T MRI scanner (Discovery MR 750, GE Healthcare, Waukesha, WI, United States). Our liver MRI protocol included the following sequence: (1) Axial T_2-weighted imaging with fat suppression: Repetition time (TR)/echo time (TE), 13000/75 msec; field of view (FOV), 360 mm × 360 mm; matrix, 320 × 320; slice thickness, 5 mm; (2) dual-echo (in-phase and opposed-phase) T_1-weighted imaging: TR/TE, 3.7/1.7 msec; FOV, 360 mm × 288 mm; matrix, 260 × 224; slice thickness, 5 mm; (3) diffusion-weighted imaging (DWI) and apparent diffusion coefficient (ADC): TR/TE, 8000/50 msec; FOV, 360 mm × 288 mm; matrix, 128×96; and slice thickness, 5 mm. DWI was obtained using respiratory triggering, a single-shot echo-planar imaging pulse sequence with b values of 0 and 800 s/mm^2; and (4) The dynamic contrast-enhanced (DCE) three-dimensional fast-spoiled gradient-recalled echo sequences were as follows: TR/TE, 3.7/1.7 msec; FOV, 360 mm × 288 mm; matrix, 260 × 224; and slice thickness, 5 mm. The DCE sequences were acquired at 15-20 s (arterial phase, AP), 50-55 s (portal phase, PP) and 85-90 s (delayed phase, DP) after contrast-agent injection.

Clinical and radiological data

Clinical data included age, sex, hepatitis B surface antigen (HBsAg) status (positive or negative), serum alpha-fetoprotein (AFP) level (recorded as > 400 μg/L or ≤ 400 μg/L), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), platelet count (PLT), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), albumin, aspartate transaminase (AST), alanine transferase (ALT) and AST/ALT.

Radiological data of all patients were retrospectively analysed by two radiologists who were unaware of the clinicopathological findings and included liver cirrhosis, tumour size, tumour shape, intratumor fat, intratumor necrosis, intratumor haemorrhage, enhancing capsule and tumour-to-liver ADC ratio. For qualitative data, an agreement was reached by negotiation when there was disagreement between the two radiologists. For quantitative data, the measurements from these two radiologists were averaged. Detailed descriptions of the radiological features are shown in Supplementary Table 1.

Pathological data

All enrolled patients underwent hepatectomy, and pathological evaluations were performed in consensus by two experienced pathologists. MTM-HCC was defined as a predominant macrotrabecular architectural pattern (> 6 cells thick) involving more than 50% of the entire tumour[7]. nMTM-HCC was defined as non-MTM-HCC. The following pathological features were recorded: tumour differentiation

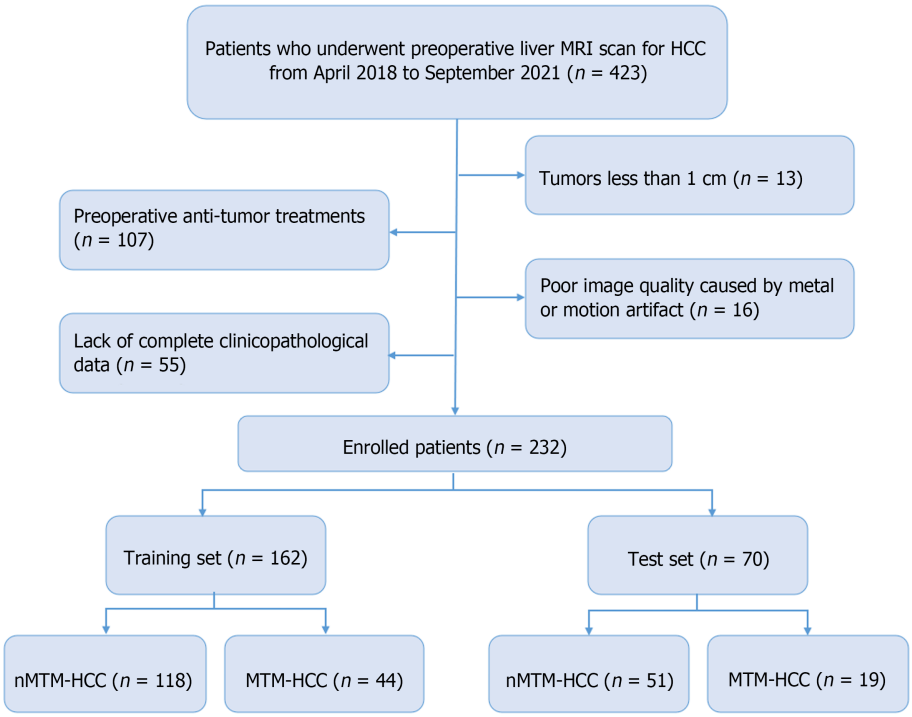

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Figure 1 The patient recruitment process. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging.

according to the Edmondson-Steiner grade (I-II or III-IV), presence of microvascular invasion, satellite nodules, and biliary invasion.

Radiomics features

Image segmentation: Image segmentation was performed with an open-source software named ITK-SNAP. The volume of interest (VOI) was defined by manually outlining the whole tumour border in AP, PP, and DP sequences slice-by-slice for each patient by a radiologist with five years of experience in liver MRI. Then, the segmentation results were validated by another radiologist with more than 10 years of experience in liver MRI using the intraclass correlation coefficient (ICC) on a cohort of 30 randomly selected patients. The image segmentation process is shown in Supplementary Figure 1.

Radiomics feature extraction: All segmented VOIs were loaded into the Pyradiomics-based PHIgo software (GE Healthcare, V1.2.0, China) for feature extraction, which complies with the image biomarker standardization initiative (IBSI)[16]. Before that, all images were subjected to standardized preprocessing, including resampling the images at the same resolution (1 mm × 1 mm × 1 mm) and classifying the grayscale into 1-10 Levels. Then, 1037 radiomics features were extracted from each VOI of each sequence, including the first-order features (first order: 18 features), shape-based features (shape: 14 features), gray-level run length matrix features (GLRLM: 16 features), gray-level size zone matrix (GLSZM: 16 features), neighbourhood gray tone difference matrix (NGTDM: 5 features), gray-level co-occurrence matrix features (GLCM: 24 features), gray-level dependence matrix (GLDM: 14 features), LoG transform features (LoG: 186 features), and wavelet transform features (wavelet: 744 features). The DCE sequences, including AP, PP, and DP, were scanned, affording 3111 radiomics features per patient.

Radiomics feature selection: The ICCs of the measurements from the two radiologists were applied to evaluate the interobserver reliability and reproducibility. The features with ICCs > 0.80 were considered robust features. Then, dimension reduction was performed using analysis of variance, correlation analysis, and gradient boosting decision tree (GBDT) to reduce data redundancy and to further select the most significant radiomics features based on DCE sequences.

Radiomics signature development: Five machine learning algorithms, including logistic regression (LR), K-nearest neighbour (KNN), Bayes, Tree, and support vector machine (SVM), were used to construct radiomics signatures based on the retained significant features. Then, we used the relative standard deviation (RSD) and bootstrap methods to quantify the stability of these five algorithms. The algorithm with the lowest RSD represented the best stability, which was used to construct the best radiomics model. Finally, the radiomics score (rad-score) was calculated *via* a linear combination of the

remaining features that were weighted by their respective coefficients to quantify the discriminability of the radiomics model. Details of the RSD can be found in the Supplementary materials.

Model construction and evaluation

Univariate logistic analysis was performed on variables, including the abovementioned clinical features, radiological features, and rad-score. Variables with $P < 0.05$ were included in the multivariate logistic analysis to determine the potential independent predictors of MTM-HCC, based on which combined model was built. In addition, the clinical model was constructed based on the final selected clinical features, and the radiological model was constructed based on the final selected radiological features. To verify the improvement in the performance of the model after including radiomics, we integrated the selected independent predictors to construct different fusion models. The area under the receiver operating characteristic (ROC) curve (AUC) and the DeLong test were used to evaluate the performance of the different models for predicting MTM-HCC. The Hosmer–Lemeshow test was used to assess the goodness-of-fit of the combined model. A nomogram based on the combined model was established for easy use to generate a probability of MTM-HCC. Then, the patients were classified into high-risk and low-risk groups according to the nomogram. The flowchart of the model construction and evaluation is shown in Figure 2.

Statistical analysis

Statistical analyses were performed with SPSS software (version 24.0, Chicago, IL, United States) and R software (version 3.4.1, Vienna, Austria). Continuous variables were expressed as the mean ± SD or median (interquartile range). Categorical variables are presented as numbers (percentages). Continuous variables were analysed using a two-sample *t* test or Mann-Whitney *U* test if not normally distributed. The chi-squared test or Fisher's exact test was used for categorical variables. Statistical significance was set at a two-sided $P < 0.05$.

RESULTS

Patient characteristics

Clinical, pathological, and radiological features of the HCC patients are shown in Tables 1 and 2. In terms of the MTM-HCC and nMTM-HCC groups, age, PLT, tumour size, intratumor haemorrhage, and tumour-to-liver ADC ratio were significantly different in both the training and test sets ($P < 0.05$). In addition, AFP > 400 μg/L ($P < 0.001$), CA19-9 ($P = 0.021$), AST ($P = 0.004$), ALT ($P = 0.023$), tumour shape ($P = 0.024$), and intratumor necrosis ($P = 0.005$) also differed significantly between the two groups in the training set. The Edmondson-Steiner grade ($P = 0.048$) differed significantly between the two groups in the test set. There were no significant differences in any of the features between the training and test sets ($P > 0.05$).

Radiomics signature development

A total of 3111 radiomics features were extracted for each patient based on the DCE sequences. Then, 2417 features with ICCs > 0.80 were obtained as robust features. After feature selection by analysis of variance and correlation analysis, 75 features were selected. Following GBDT, 27 features were ultimately retained, as shown in Figure 3A. The RSD values based on LR, KNN, Bayes, Tree, and SVM were 3.8%, 8.6%, 4.3%, 17.7%, and 17.4%, respectively. Therefore, the LR machine learning algorithm was chosen to construct the best radiomics signature (Figure 3B). The corresponding rad-score was calculated and was significantly different between the MTM-HCC and nMTM-HCC groups in the training set ($P < 0.001$) and test set ($P = 0.002$), as shown in Figure 3C. Details of the retained radiomics features and rad-score are shown in Supplementary Table 2 and Supplementary Figure 2.

Model construction and comparison

The multivariate logistic analysis showed that age (OR = 0.956, $P = 0.034$), AFP (OR = 10.066, $P < 0.001$), tumour size (OR = 3.316, $P = 0.002$), tumour-to-liver ADC ratio (OR=0.156, $P = 0.037$), and rad-score (OR = 2.923, $P < 0.001$) were independent predictors of MTM-HCC (Table 3). A clinical model was constructed based on age and AFP. A radiological model was constructed based on tumour size and the tumour-to-liver ADC ratio. To verify the improvement in the performance of the model after including radiomics, we constructed different fusion models, including a clinical-radiomics model and a radiological-radiomics model. Finally, we integrated all five of the selected independent predictors to build the combined model. The corresponding model score of the combined model was calculated, as shown in Supplementary Figure 3.

Among the six different models, the combined model performed best, with AUCs of 0.896 and 0.805 in the training and test sets, respectively (Table 4). The DeLong test showed that the predictive performances of the clinical-radiomics model and radiological-radiomics model were significantly improved compared to those of the clinical model (AUCs: 0.888 *vs* 0.836, $P = 0.046$) and radiological

Table 1 Clinical and pathological features in the training and test sets

Characteristics	Training set (*n* = 162)			Test set (*n* = 70)			*P* value
	nMTM-HCC (*n* = 118)	MTM-HCC (*n* = 44)	*P* value	nMTM-HCC (*n* = 51)	MTM-HCC (*n* = 19)	*P* value	
Age, mean ± SD	60.6 ± 10.9	52.4 ± 9.9	< 0.001	60.7 ± 10.4	54.7 ± 9.7	0.034	0.647
Sex (men, %)	102 (86.4)	42 (95.5)	0.179	39 (76.5)	18 (94.7)	0.161	0.125
HBsAg (positive, %)	91 (77.1)	33 (75.0)	0.777	38 (74.5)	14 (73.7)	0.944	0.712
AFP > 400 μg/L, *n* (%)	18 (15.3)	31 (70.5)	< 0.001	12 (23.5)	9 (47.4)	0.053	0.970
CEA (ug/L)	2.6 (2.1)	2.7 (1.9)	0.719	2.2 (2.0)	2.2 (1.2)	0.543	0.332
CA19-9 (U/mL)	15.0 (19.3)	28.0 (25.4)	0.021	16.7 (17.1)	16.0 (34.4)	0.687	0.770
PLT (× 10^9/L)	135.0 (83.0)	174.0 (88.5)	0.027	135.0 (57.0)	175.0 (127.0)	0.001	0.970
TBIL (μmol/L)	16.6 (10.8)	20.7 (9.8)	0.067	15.7 (10.3)	14.4 (7.6)	0.916	0.509
DBIL (μmol/L)	3.8 (2.6)	4.8 (2.9)	0.060	3.2 (2.1)	3.3 (2.7)	0.620	0.419
IBIL (μmol/L)	12.5 (7.0)	15.0 (7.4)	0.136	12.2 (7.8)	10.9 (4.5)	0.712	0.061
Albumin, mean ± SD	37.8 ± 5.0	37.0 ± 5.8	0.387	37.8 ± 5.0	38.3 ± 5.1	0.715	0.647
AST (U/L)	32.0 (23.5)	43.0 (68.8)	0.004	33.0 (29.0)	43.0 (61.0)	0.135	0.980
ALT (U/L)	31.5 (34.0)	46.0 (64.0)	0.023	28.0 (35.0)	57.0 (96.0)	0.074	0.748
AST/ALT	1.1 (0.6)	1.1 (0.5)	0.702	1.3 (0.5)	1.1 (0.7)	0.207	0.842
Edmondson-Steiner grade (III-IV, %)	38 (32.2)	18 (40.9)	0.300	14 (27.5)	10 (52.6)	0.048	0.967
Microvascular invasion, *n* (%)	56 (47.5)	20 (45.5)	0.820	18 (35.3)	10 (52.6)	0.188	0.331
Satellite nodules, *n* (%)	9 (7.6)	5 (11.4)	0.452	1 (2.0)	1 (5.3)	0.461	0.110
Biliary invasion, *n* (%)	4 (3.4)	1 (2.3)	0.715	2 (3.9)	1 (5.3)	0.805	0.646

Data in parentheses are presented as median (interquartile range) or *n* (%). MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; PLT: Platelet count; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; AST: Aspartate transaminase; ALT: Alanine transferase; SD: Standard deviation.

model (AUCs: 0.796 *vs* 0.688, $P = 0.012$), respectively, in the training set, highlighting the improved predictive performance of radiomics. In addition, the combined model performed better than the radiological model ($P < 0.001$), radiomics model ($P = 0.001$), and radiological-radiomics model ($P = 0.002$) in the training set, and the combined model was significantly different from the clinical model in both the training set ($P = 0.023$) and the test set ($P = 0.042$) (Table 4 and Figure 4).

Nomogram building and verification

Based on the combined model, we developed an intuitive, simple-to-use nomogram for individual risk prediction of MTM-HCC (Figure 5). The Hosmer–Lemeshow test exhibited good calibration of the nomogram in the training set ($P = 0.995$) and test set ($P = 0.466$). According to the optimal cut-off value of -1.663, the patients were stratified into low-risk and high-risk groups. As shown in Figure 6, there were significant differences in the number of patients who were predicted to have MTM-HCC between the low-risk and high-risk groups in the training set ($P < 0.001$), test set ($P = 0.003$), and all study populations ($P < 0.001$), indicating the clinical applicability of the nomogram.

DISCUSSION

In this study, we comprehensively evaluated clinical and radiological features and found that age, AFP, tumour size, and tumour-to-liver ADC ratio were significant independent predictors of MTM-HCC. By comparing five different machine learning algorithms (LR, KNN, Bayes, Tree, and SVM), we finally selected the LR algorithm with the best stability to construct the radiomics signature. Finally, by further comparing the predictive performance of the different models, the optimal combined model was

Table 2 Radiological features of patients in the training and test sets

Radiological features	Training set (*n* = 162)			Test set (*n* = 70)			*P* value
	nMTM-HCC (*n* = 118)	MTM-HCC (n = 44)	*P* value	nMTM-HCC (*n* = 51)	MTM-HCC (*n* = 19)	*P* value	
Liver cirrhosis (positive, %)	69 (58.5)	29 (65.9)	0.389	34 (66.7)	15 (78.9)	0.319	0.168
Tumour size > 5 cm, *n* (%)	29 (24.6)	22 (50.0)	0.002	12 (23.5)	10 (52.6)	0.020	0.994
Tumour shape (irregular, %)	25 (21.2)	17 (38.6)	0.024	17 (33.3)	7 (36.8)	0.783	0.195
Intratumor fat, *n* (%)	24 (20.3)	10 (22.7)	0.740	11 (21.6)	1 (5.3)	0.210	0.500
Intratumor necrosis, *n* (%)	34 (28.8)	23 (52.3)	0.005	13 (25.5)	8 (42.1)	0.177	0.443
Intratumor hemorrhage, *n* (%)	24 (20.3)	17 (38.6)	0.017	7 (13.7)	8 (42.1)	0.025	0.526
Enhancing capsule, *n* (%)	85 (72.0)	35 (79.5)	0.332	39 (76.5)	15 (78.9)	0.826	0.620
Tumour-to-liver ADC ratio	0.9 (0.2)	0.8 (0.3)	0.018	0.9 (0.1)	0.8 (0.2)	0.045	0.312

Data in parentheses are presented as median (interquartile range) or *n* (%). MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma; ADC: Apparent diffusion coefficient.

Table 3 Results of univariate and multivariate logistic regression analyses

Variables	Univariate logistic regression		Multivariate logistic regression	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Clinical features				
Age	0.930 (0.896, 0.964)	< 0.001	0.956 (0.918, 0.997)	0.034
AFP > 400 μg/L	13.248 (5.839, 30.058)	< 0.001	10.066 (4.304, 23.541)	< 0.001
PLT	1.006 (1.002, 1.011)	0.009	NA	NA
Radiological features				
Tumour size > 5 cm	3.069 (1.487, 6.333)	0.002	3.316 (1.579, 6.962)	0.002
Tumour shape	2.342 (1.106, 4.961)	0.026	NA	NA
Intratumor necrosis	2.706 (1.326, 5.521)	0.006	NA	NA
Intratumor hemorrhage	2.466 (1.160, 5.244)	0.019	NA	NA
Tumour-to-liver ADC ratio	0.183 (0.035, 0.972)	0.046	0.156 (0.027, 0.894)	0.037
Radiomics				
Rad-score	2.718 (1.809, 4.084)	< 0.001	2.923 (1.740, 4.911)	< 0.001

AFP: Alpha-fetoprotein; PLT: Platelet count; ADC: Apparent diffusion coefficient; OR: Odds ratio; CI: Confidence interval; NA: Not available.

selected, and a visual nomogram was constructed, which provided a reliable theoretical basis for the development of a simple, easy-to-use, and accurate assessment tool and indicated that it had great potential in the field of preoperative noninvasive prediction of MTM-HCC.

Although some previous studies have focused on the clinical and radiological features of MTM-HCC, there is no consensus on the best biomarker for predicting MTM-HCC. Our study showed that MTM-HCC patients presented with large tumour sizes and high AFP loads, which were consistent with previous findings[17-20]. Interestingly, this study found that the age of patients with MTM-HCC was lower than that of the patients with nMTM-HCC. This may be because the activation of angiogenesis is the reason for the unique invasive biological characteristics of such tumours. Overexpression of angiopoietin 2 and vascular endothelial growth Factor A can cooperatively promote new angiogenesis in MTM-HCC, and these factors are indicators of poor prognosis in solid tumours[6,21]. This also confirmed that the aggressiveness of MTM-HCC may be related to molecular factors. Various studies

Table 4 Predictive performance of different models in training and test sets

Models	Training set				Test set			
	AUC (95%CI)	Accuracy	Sensitivity	Specificity	AUC (95%CI)	Accuracy	Sensitivity	Specificity
Clinical	0.836 (0.773-0.888)	0.802	0.750	0.822	0.701 (0.575-0.814)	0.671	0.579	0.706
Radiological	0.688 (0.604-0.769)	0.685	0.636	0.703	0.723 (0.610-0.829)	0.700	0.632	0.725
Radiomics	0.766 (0.692-0.836)	0.772	0.568	0.847	0.739 (0.634-0.837)	0.743	0.579	0.804
Clinical-radiomics	0.888 (0.835-0.934)	0.802	0.909	0.763	0.793 (0.682-0.893)	0.686	0.737	0.667
Radiological-radiomics	0.796 (0.725-0.858)	0.772	0.636	0.822	0.764 (0.661-0.859)	0.729	0.632	0.765
Combined	0.896 (0.847-0.939)	0.796	0.932	0.746	0.805 (0.704, 0.895)	0.700	0.895	0.628

AUC: Area under the receiver operating characteristic curve; CI: Confidence interval.

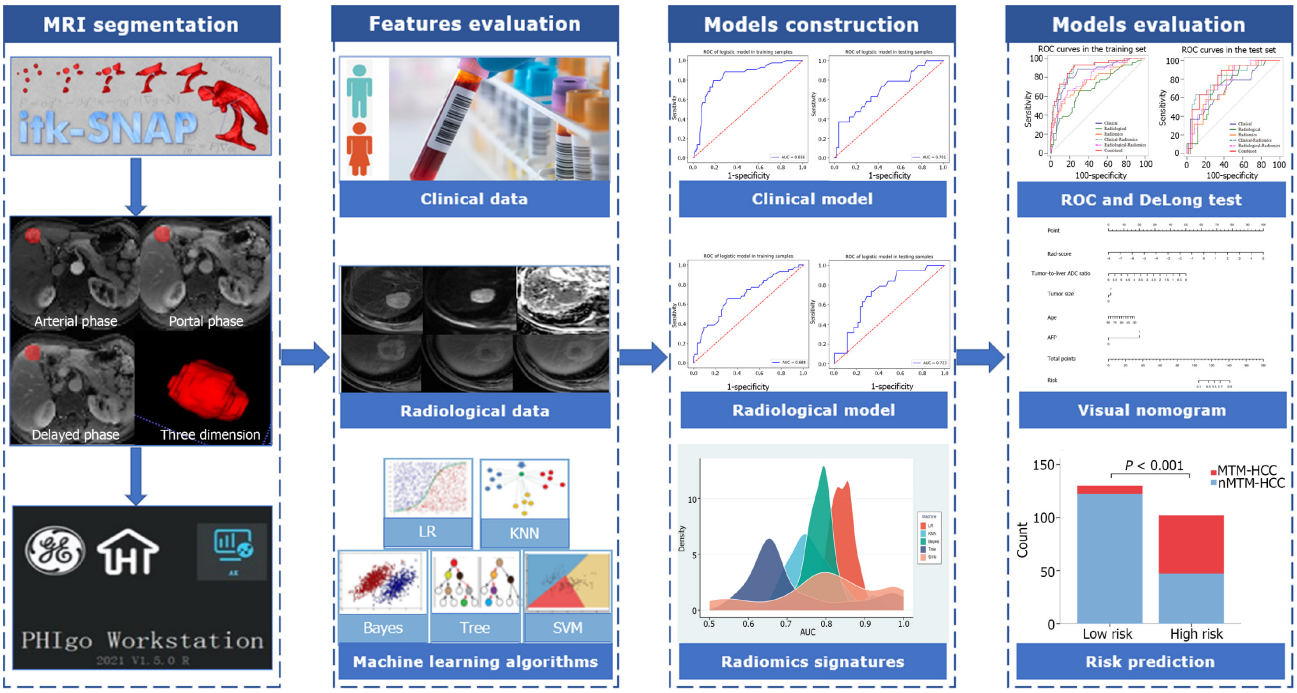

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Figure 2 The radiomics workflow. LR: Logistic regression; KNN: K-nearest neighbour; SVM: Support vector machine; ROC: Receiver operating characteristic; MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

have explored the relationship between PLT and MTM-HCC, but no consensus has been reached[22]. Our study showed that PLT was not an independent predictor. The multivariate logistic analysis showed that a low tumour-to-liver ADC ratio was an independent predictor of MTM-HCC, which was consistent with the results of Chen *et al*[12]. This is because the cellular structure is increased, the arterial supply is reduced, and diffusion is more restricted in more aggressive HCC[5]. In addition, previous studies[11,20] have suggested that intratumor necrosis can be used as an independent predictor of MTM-HCC. However, in our study, although the univariate logistic analysis showed that intratumor necrosis was more common and statistically significant in MTM-HCC, it was not an independent predictor, which was also consistent with the results of Zhu *et al*[14]. This may be because intratumor necrosis is not a commonly reported finding of aggressiveness or poor prognosis in HCC and lacks high specificity. This finding was more common in non-HCC malignancies, such as intrahepatic cholangiocarcinoma or metastasis, and therefore was considered a feature of non-HCC malignancies[23].

At present, there is no research on the value of different machine learning algorithms in predicting MTM-HCC. In this study, we compared the performance of radiomics signatures constructed by five machine learning algorithms, and the results showed that the radiomics signature constructed by the LR algorithm had the best stability, with an RSD of 3.8%. The radiomics model based on the LR algorithm performed well in predicting MTM-HCC, with AUCs of 0.766 and 0.739 and specificities of 0.847 and 0.804 in the training and test sets, respectively. Therefore, radiomics is very useful for timely capturing

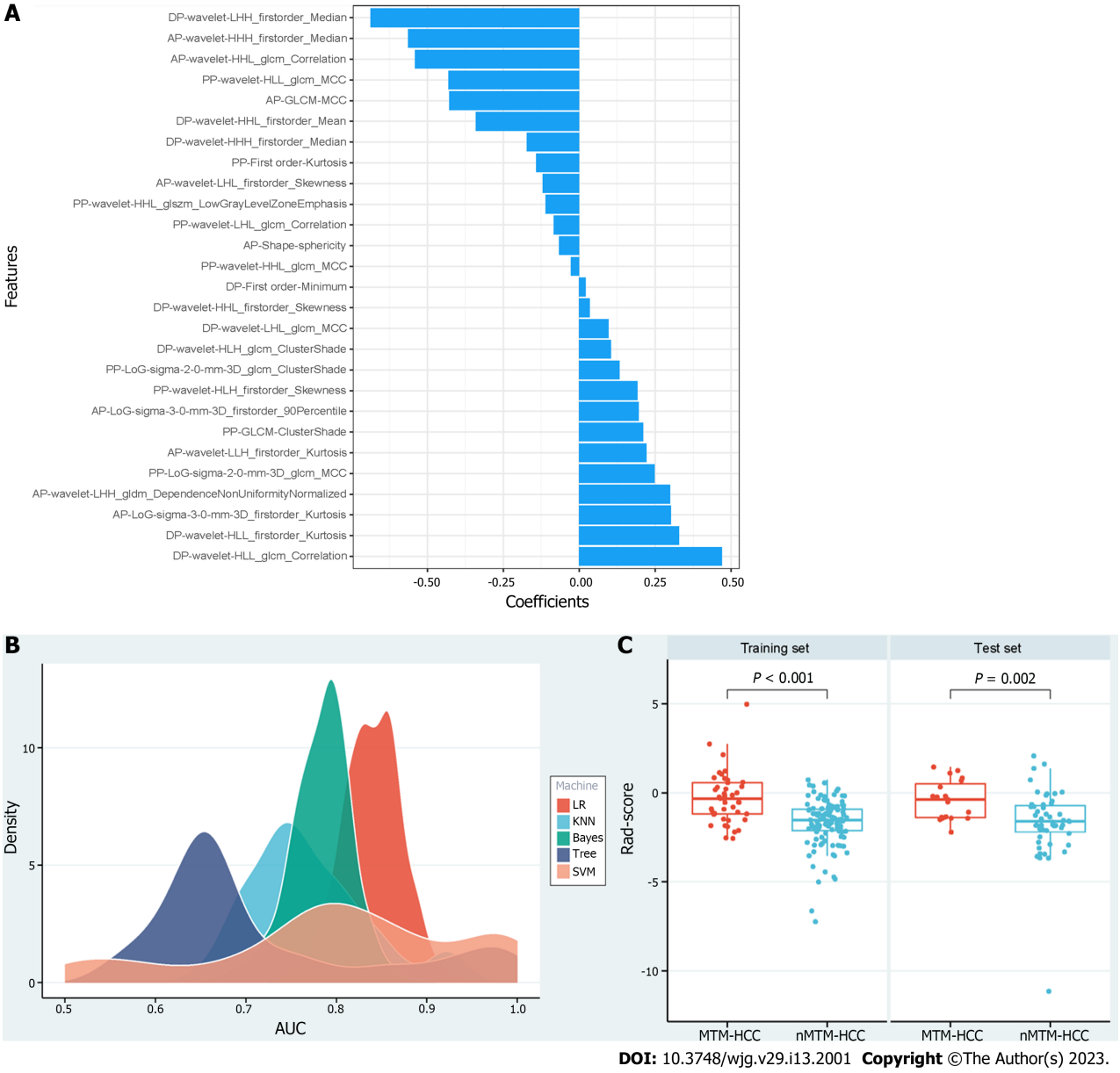

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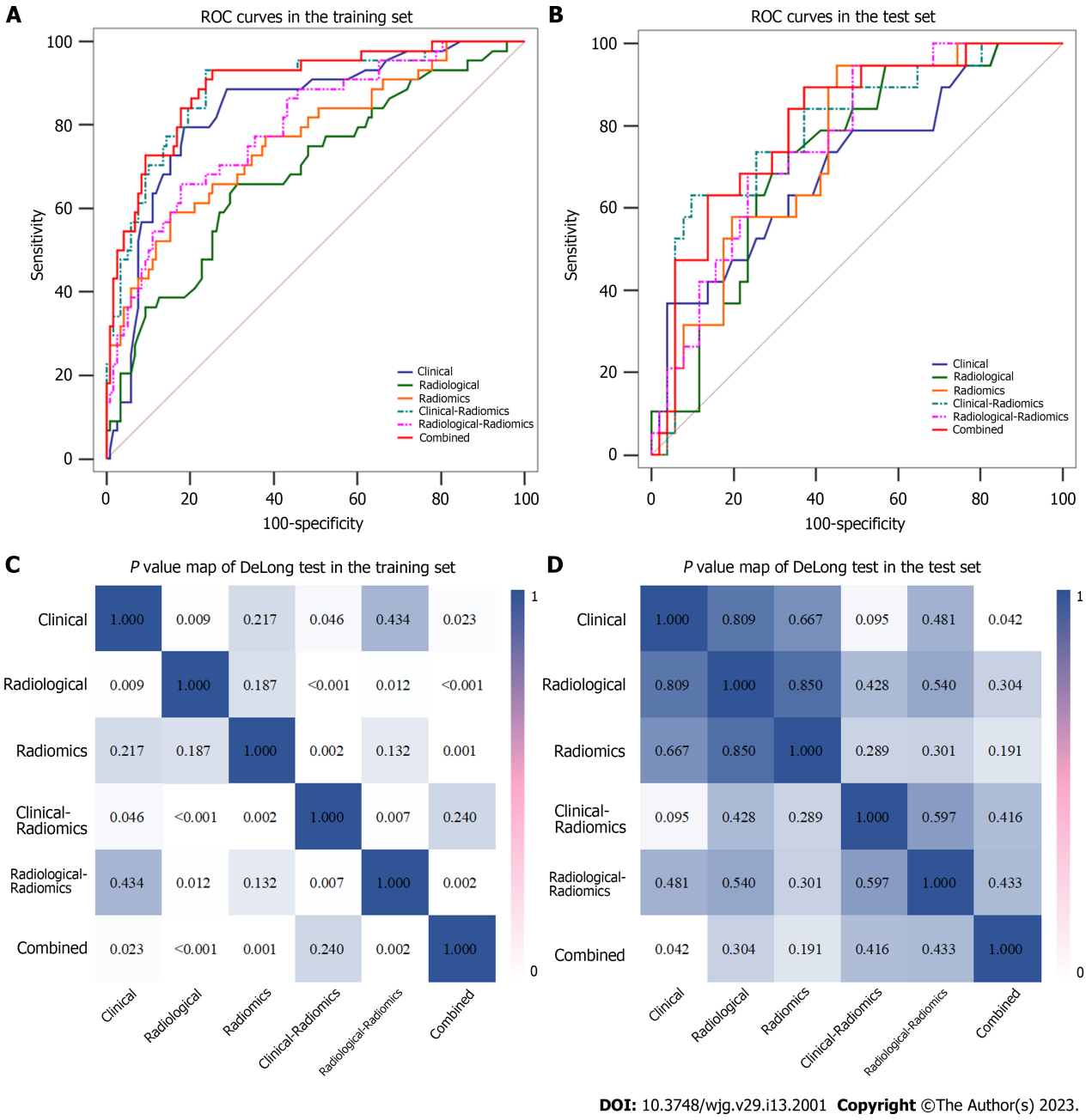
Figure 3 Radiomics signature development. A: Of 27 selected radiomics features and their coefficients; B: Density distribution of the area under the receiver operating characteristic curve of the radiomics signatures constructed by the five machine learning algorithms; C: Box-scatter plots show that rad-score of macrotrabecular-massive hepatocellular carcinoma is substantially higher than that of non-macrotrabecular-massive hepatocellular carcinoma in both the training and test sets. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

and reflecting the underlying histopathological features. In addition, 27 features were finally retained in this study, of which 22 (81.5%) features (4 LoG and 18 wavelet features) were extracted from the original and derived images[24]. For example, this study confirmed that "cluster shade", "correlation" and "MCC" were the most meaningful features among GLCM, showing differences in the regional signal intensity distribution and linear dependency. Among the first-order features, “Mean” and “Median” describe the mean and median grey intensity of the voxel intensity within the tumour region, respectively, and the differences in the grey intensity distribution are represented by “Kurtosis” and “Skewness”. “GISZM_LowGrayLevelZoneEmphasis” and “GLDM_DependenceNonUniformityNormalized” represent the heterogeneity of the tumour. These results are consistent with some radiomics features extracted from previous studies on the pathological and survival prediction of HCC[25-27], indicating that LoG and wavelet features can represent signal intensity distribution or grey distribution in tissues, which can better reflect the biological characteristics and heterogeneity of tumours.

Our study found that the clinical model based on age and AFP (AUC, 0.836; sensitivity, 0.750; specificity, 0.822) had a significantly higher diagnostic efficiency than the clinical model based on AST, PT, and AFP proposed by Shan *et al*[28] (AUC, 0.723; sensitivity, 0.711; specificity, 0.607) in predicting MTM-HCC. The limitation of our clinical model was that it had poor performance in the test set, with an AUC of 0.701, a sensitivity of 0.579, and a specificity of 0.706. However, when the radiomics signature was introduced, the predictive performances of the clinical-radiomics model and radiological-radiomics

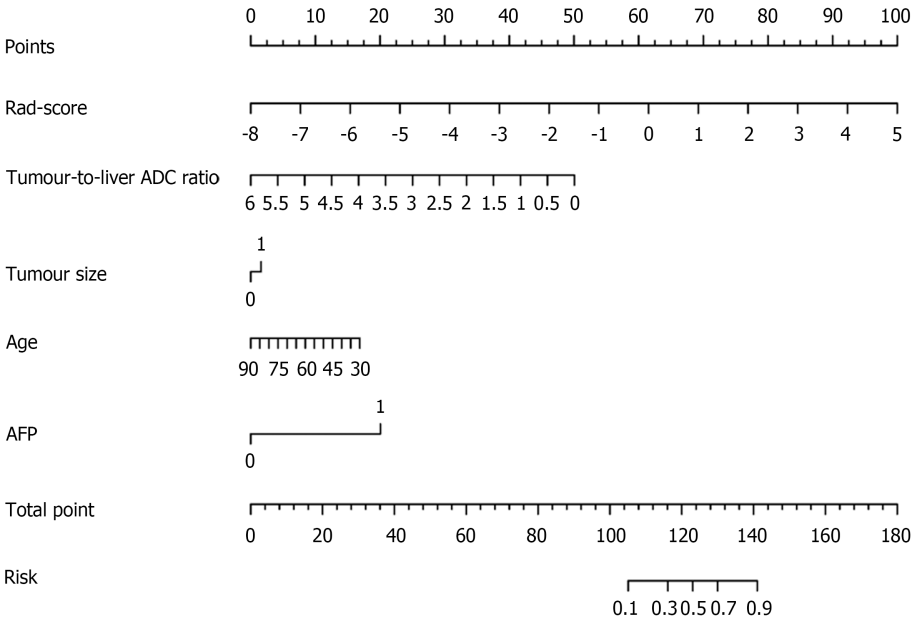


DOI: 10.3748/wjg.v29.i13.2001

Figure 4 Predictive performance of different models. A and B: Receiver operating characteristic curves of different models in the training and test sets; C and D: *P* value map of DeLong test between the different models in the training and test sets. ROC: Receiver operating characteristic.

model were improved compared to the clinical model (AUCs: 0.888 *vs* 0.836 in the training set, 0.793 *vs* 0.701 in the test set) and radiological model (AUCs: 0.796 *vs* 0.688, 0.764 *vs* 0.723 in the test set), respectively, highlighting the improved predictive performance of radiomics. This is due to the unprecedented opportunities for extracting potential quantitative information from images provided by advanced radiomics analysis. Traditional imaging diagnosis usually relies on morphological changes observed by the naked eye. However, it often takes a long time for the appearance of observable morphological image changes of tumours caused by pathological changes. This also indicates that it is valuable for clinical applications to extract the quantitative radiomics features behind the images.

Since the diagnosis of HCC does not require biopsy, preoperative noninvasive identification of the MTM-HCC subtype is critical for treatment and prognosis. There was only one previous study on radiomics in the prediction of MTM-HCC, and the nomogram established based on radiomics and intratumor fat showed satisfactory prediction performance, with an AUC of 0.785[14]. However, that small sample study (n = 88) lacked a test set, and the reproducibility of the results was limited. It is worth mentioning that our nomogram performed best in the preoperative noninvasive prediction of MTM-HCC, with AUCs of 0.896 and 0.805 in the training and test sets, respectively. This study achieves the first step towards the noninvasive evaluation of MTM-HCC using radiomics and clinical and radiological features in clinical practice and may guide the selection of patients for whom targeted



DOI: 10.3748/wjg.v29.i13.2001

Figure 5 Visual nomogram construction. ADC: Apparent diffusion coefficient; AFP: Alpha-fetoprotein.

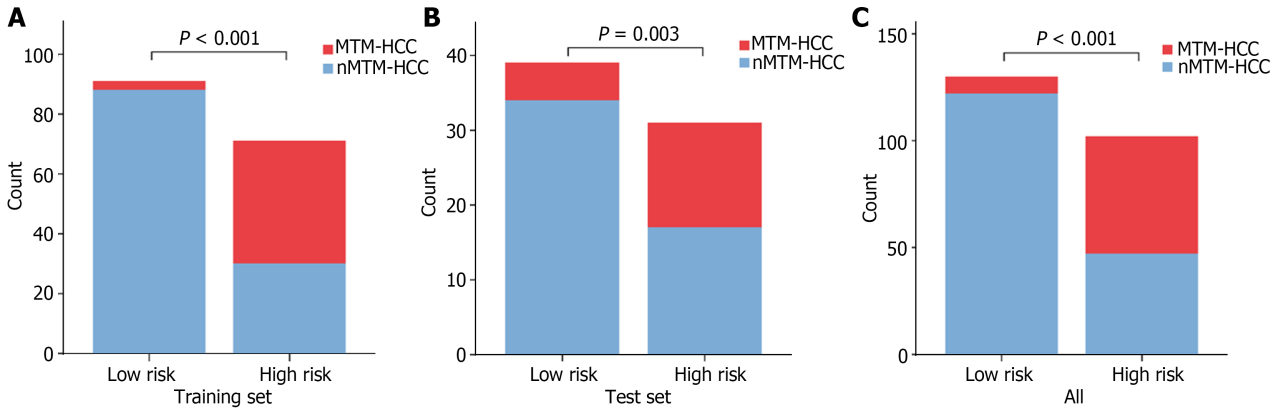


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Figure 6 Risk prediction based on the nomogram. Patients were divided into high-risk and low-risk groups according to the nomogram. The probability of pathological macrotrabecular-massive hepatocellular carcinoma in the high-risk group was significantly higher than that in the low-risk group in the training set, test set, and all study populations. A: Training set; B: Test set; C: All study populations. MTM-HCC: Macrotrabecular-massive hepatocellular carcinoma; nMTM-HCC: Non-macrotrabecular-massive hepatocellular carcinoma.

therapies would be effective. This again suggested the application value of radiomics in predicting MTM-HCC. Integrating multidimensional features is of great significance for building a powerful prediction model.

Some limitations should be noted. First, this retrospective study only included surgically resected lesions, which may cause selection bias. Therefore, the results of this study may not be representative of the entire clinical spectrum. Second, all patients were recruited from a single center, which may limit the external validation. Therefore, further studies using larger, multicenter samples are needed to verify our findings.

CONCLUSION

In this study, we found that age, AFP, tumour size, and tumour-to-liver ADC ratio were significant independent predictors of MTM-HCC. We compared and selected the optimal LR machine learning algorithm to construct the radiomics signature. The nomogram showed excellent predictive ability in preoperatively identifying MTM-HCC and showed great potential in clinical application, which was helpful to guide individualized treatment and improve the long-term survival outcomes of HCC

patients.

ARTICLE HIGHLIGHTS

Research background

Macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) shows an aggressive phenotype. Early diagnosis of MTM-HCC is beneficial to prevent early recurrence and improve prognosis. Radiomics can convert medical images into high-throughput quantification features, which greatly push the development of precision medicine.

Research motivation

Currently, magnetic resonance imaging (MRI) features have been successfully applied to identify MTM-HCC but have mainly focused on the qualitative analysis of imaging features. In this study, we systematically analysed radiomics, clinical and radiological features to build a more comprehensive prediction model. We aimed to develop a noninvasive model for the preoperative prediction of MTM-HCC.

Research objectives

In this study, we aimed to establish and verify a nomogram based on contrast-enhanced MRI for preoperatively identifying MTM-HCC by comparing different machine learning algorithms.

Research methods

A total of 232 (training set, 162; test set, 70) hepatocellular carcinoma patients were enrolled. Radiomics features were extracted from contrast-enhanced MRI, followed by dimension reduction. Logistic regression (LR), K-nearest neighbour, Bayes, Tree, and support vector machine algorithms were used to construct radiomics signatures. The relative standard deviation (RSD) was used to quantify the stability of these five algorithms. Multivariable logistic analysis was used to select the useful clinical and radiological features, and different predictive models were established. The performances of the different models were assessed using the area under the curve (AUC).

Research results

The LR algorithm with the smaller RSD (3.8%) was used to construct the best radiomics signature, which performed well with AUCs of 0.766 and 0.739 in the training and test sets, respectively. Age, alpha-fetoprotein, tumour size, tumour-to-liver apparent diffusion coefficient ratio, and radiomics score were identified as independent predictors of MTM-HCC to build the nomogram, which performed best with AUCs of 0.896 and 0.805 in the training and test sets, respectively.

Research conclusions

The nomogram is a reliable tool for preoperatively identifying the MTM-HCC subtype.

Research perspectives

More precise and reliable tools are urgently needed to predict the MTM-HCC subtype. Radiomics is a new method to convert medical images into high-throughput quantification features. In this study, we aimed to develop a dynamic contrast-enhanced MRI-based nomogram for preoperatively identifying the MTM-HCC subtype.

FOOTNOTES

Author contributions: Shi LL was the guarantor and designed the study; Zhang Y, He D, Liu J, and Shi LL participated in the acquisition, analysis, and interpretation of the data; Wei YG reviewed statistical methods; Zhang Y drafted the initial manuscript; Shi LL revised the article critically for important intellectual content; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Changes in characteristics of patients with hepatitis C virus-related cirrhosis from the beginning of the interferon-free era

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Abstract

BACKGROUND

Nearly 290000 patients with chronic hepatitis C die annually from the most severe complications of the disease. One of them is liver cirrhosis, which occurs in about 20% of patients chronically infected with the hepatitis C virus (HCV). Direct-acting antivirals (DAAs), which replaced interferon (IFN)-based regimens, significantly improved the prognosis of this group of patients, increasing HCV eradication rates and tolerability of therapy. Our study is the first to assess changes in patient profile, effectiveness, and safety in the HCV-infected cirrhotic population in the IFN-free era.

AIM

To document changes in patient characteristics and treatment regimens along with their effectiveness and safety profile over the years.

METHODS

The studied patients were selected from 14801 chronically HCV-infected individuals who started IFN-free therapy between July 2015 and December 2021 in 22 Polish hepatology centers. The retrospective analysis was conducted in real-world clinical practice based on the EpiTer-2 multicenter database. The measure of treatment effectiveness was the percentage of sustained virologic response (SVR) calculated after excluding patients lost to follow-up. Safety data collected during therapy and the 12-wk post-treatment period included information on adverse events, including serious ones, deaths, and treatment course.

RESULTS

The studied population (n = 3577) was balanced in terms of gender in 2015-2017, while the following years showed the dominance of men. The decline in the median age from 60 in 2015-2016 to 57 years in 2021 was accompanied by a decrease in the percentage of patients with comorbidities and comedications. Treatment-experienced patients dominated in 2015-2016, while treatment-naive individuals gained an advantage in 2017 and reached 93.2% in 2021. Genotype (GT)-specific options were more prevalent in treatment in 2015-2018 and were supplanted by pangenotypic combinations in subsequent years. The effectiveness of the therapy was comparable regardless of the period analyzed, and patients achieved an overall response rate of 95%, with an SVR range of 72.9%-100% for the different therapeutic regimens. Male gender, GT3 infection, and prior treatment failure were identified as independent negative predictors of therapeutic success.

CONCLUSION

We have documented changes in the profile of HCV-infected cirrhotic patients over the years of accessibility to changing DAA regimens, confirming the high effectiveness of IFN-free therapy in all analyzed periods.

Key Words: Hepatitis C; Liver cirrhosis; Direct-acting antivirals; Pangenotypic; Genotype-specific; Epidemiology

Core Tip: Patients with cirrhosis in the course of chronic infection with the hepatitis C virus, in whom the risk of death due to advanced liver disease is the highest, seem to be the greatest beneficiaries of the introduction of therapies with direct-acting antiviral drugs. Our analysis tracking changes in the profile of these patients documents the very high effectiveness and good safety profile from the beginning of the interferon-free era to the present.

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INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) remains a global health problem, affecting 58 million people worldwide, according to the most recent estimates of the World Health Organization (WHO) published in 2022[1]. Despite its initially asymptomatic course, untreated chronic hepatitis C (CHC) can lead to progressive liver disease resulting in cirrhosis. Among HCV-infected patients, about 20% are at risk of developing cirrhosis after an average of about 20 years of infection[2], and 3%-6% of them develop decompensation of liver function each year[3]. Hepatic decompensation and hepatocellular carcinoma (HCC) are reported as the most severe complications of CHC, causing globally nearly 290000 deaths each year[1].

The antiviral treatment of patients with cirrhosis, as they are at risk of developing these most severe and potentially fatal complications, is crucial in achieving the WHO goal formulated in 2016, which assumes the reduction of mortality due to HCV by 65% by 2030[1]. In the era of interferon (IFN)-based antiviral regimens, therapeutic options were severely limited due to contraindications and poor safety profile in this patient population[4]. Even the introduction in 2011 of the first direct-acting antivirals (DAAs) and HCV protease inhibitors (telaprevir and boceprevir), which were registered for use with pegylated IFN, was not a breakthrough for this group of patients due to the limitations still associated with IFN[5]. Only the availability of IFN-free therapies based exclusively on a combination of DAA agents has significantly improved safety parameters and treatment efficacy, representing a real revolution in managing HCV-infected patients with cirrhosis, including those with decompensation[6]. As a result, at the beginning of the DAA era, cirrhotic patients were prioritized in access to treatment, with some countries even limiting therapy reimbursement to this group of patients[7]. The findings of a number of clinical trials and real-world experience (RWE) studies involving this particular patient population published to date indicate that, despite the lack of contraindications to DAA therapy, the presence of cirrhosis is still considered an unfavorable factor for achieving a sustained virologic response (SVR)[8,9].

Recently, several RWE studies have been conducted in different world parts, summarizing the effectiveness of DAA treatment and changes in demographic and clinical characteristics in HCV-infected patients treated with DAA regimens[10-14]. However, none of these have focused on cirrhotic patients, while filling key knowledge gaps regarding this population could support the WHO's goal of eliminating HCV as a major public health burden by 2030.

Therefore, the present study aimed to track changes in the profile of HCV-infected patients with cirrhosis treated with DAA options, along with documenting the evolution of antiviral regimens in RWE practice over seven years of access to IFN-free therapy. To this end, a retrospective analysis of the Polish population of HCV-infected patients with cirrhosis who were treated between 2015 and 2021 was conducted.

MATERIALS AND METHODS

Study population

The study population was selected from 14801 adult patients infected with HCV who started IFN-free antiviral treatment in 22 Polish hepatology centers from the beginning of the DAA availability between July 1, 2015 and December 31, 2021. The analysis was a part of the EpiTer-2 database, a retrospective national study evaluating antiviral therapy of HCV-infected patients in routine clinical practice, supported by the Polish Association of Epidemiologists and Infectiologists. The present study consisted of all consecutive CHC patients with liver cirrhosis treated with IFN-free therapy, reimbursed by the Polish National Health Fund (NFZ). Since the beginning of the availability of DAA regimens in Poland, there have been no restrictions related to the severity of liver disease or history of previous therapy in the qualification of patients for antiviral treatment. The choice of regimen, dose, and length of treatment course was at the discretion of the treating physician based on the available therapeutic options, and treatment was administered following the product characteristics, the protocol of the NFZ therapeutic program, and the recommendations of the Polish Group of Experts for HCV[15-19]. Before starting treatment, the patient signed a consent form as required by the current regulations of the therapeutic program.

Data collection

The data were collected retrospectively with an online questionnaire operated by Tiba LLC based on the medical records. The study was carried out by comparison of six groups of patients diagnosed with liver cirrhosis who were divided based on the time of treatment initiation: 2015-2016, 2017, 2018, 2019, 2020, and 2021. Parameters gathered at baseline included demographic and clinical data: Gender, age, body mass index, HCV genotype (GT), comorbidities, concomitant medications, information on the severity of the liver disease, coinfections of the human immunodeficiency virus (HIV) and hepatitis B virus (HBV), and history of previous antiviral therapy. Baseline laboratory parameters were recorded, including serum alanine transaminase activity, bilirubin concentrations, albumin, creatinine, hemoglobin, platelet count, and HCV viral load. The patient groups were compared regarding the treatment regimens used and their efficacy and safety outcomes.

Assessment of liver disease severity

The advancement of liver disease was evaluated by assessing liver stiffness using real-time shear wave elastography with an Aixplorer (SuperSonic Imagine, Aix-en-Provence, France) or transient elastography with the usage of FibroScan (Echosens, France). Based on the METAVIR score, according to the European Association for the Study of the Liver guidelines, the cutoff value of 13 kPa was used for the prediction of individuals with F4 who were considered to be cirrhotic[20]. The patients were scored on Child-Pugh (CP) and model for end-stage liver disease, and data on the presence of esophageal varices, past or present hepatic decompensation and the history of HCC, and liver transplantation were collected. Patients who scored as B or C on the CP scale were considered decompensated.

Efficacy assessment

The efficacy endpoint of the study was SVR. It was defined as undetectable HCV RNA at least 12 wk after completion of treatment. Patients with detectable HCV RNA at this time point were identified as virologic non-responders, whereas those with no HCV RNA assessment 12 wk after the end of treatment were considered lost to follow-up. Depending on local practices at the testing site, the concentration of HCV RNA was measured using COBAS TaqMan HCV v2.0 (Roche Molecular Diagnostics, Pleasanton, CA, United States), COBAS AmpliPrep HCV (Roche Molecular Diagnostics, Pleasanton, CA, United States), the m2000 Real-Time System (Abbott Molecular, Des Plaines, IL, United States), or the Xpert HCV Viral Load real-time assay (Cepheid, Sunnyvale, California, United States).

Safety assessment

Through treatment and 12 wk after its completion, the following safety data were collected: The occurrence of adverse events (AEs), including severe AEs, and death, as well as the rates of modification or discontinuation of the therapy course. In addition, AEs of special interest related to the deterioration of liver function involving gastrointestinal bleeding, ascites, and encephalopathy were reported.

Ethics

The data were originally collected not for scientific purposes but to evaluate treatment efficacy and safety in real-world settings with registered medications. Patients were not exposed to any experimental interventions. According to the local law (the Polish Pharmaceutical Law of 6 September 2001, art. 37al), noninterventional studies do not require ethics committee approval. Due to the retrospective design of the analysis, additional consent from patients was not required, but as mentioned, they signed a consent form to enter the therapeutic program. Data of patients were collected and analyzed following applicable data protection rules.

Statistical analysis

Categorical data are presented as numbers and percentages, whereas continuous data are expressed as the mean (SD) or median and interquartile ranges. The SVR was evaluated for all patients who initiated the treatment after the exclusion of those lost to follow-up as per protocol analysis. Statistical analyses were performed using Statistica v. 13 (StatSoft, Tulsa, OK, United States). Multiple logistic regression was used to predict the odds of no response to HCV treatment based on predictor variables selected through univariate analysis.

RESULTS

Patient characteristics

The studied population consisted of 3577 patients selected from 14801 individuals treated with IFN-free regimens included in the EpiTer-2 database. They were divided into six groups based on the date of treatment initiation: (1) July 1, 2015 to December 31, 2016; (2) January 1, 2017 to December 31, 2017; (3) January 1, 2018 to December 31, 2018; (4) January 1, 2019 to December 31, 2019; (5) January 1, 2020 to

December 31, 2020; and (6) January 1, 2021 to December 31, 2021 (Figure 1).

Between the first period analyzed and 2018, the percentage of cirrhotic patients treated with antiviral therapy significantly decreased from 43.7% to 14.8%, but they began to rise again in 2019 to a value of 21.3% in 2021. The analyzed population was sex-balanced until 2017, while men's predominance was observed for consecutive years. Between the first and the last time interval, a minor reduction in the median (Q1, Q3) age was documented in women: From 63 (57, 69) in 2015-2016 to 61 (53, 68.5) years in 2021 and slightly greater reduction in men age: From 57 (47, 63) in 2015-2016 to 52 (44, 63) years in 2021 (Table 1). Women were older than men, with a peak of around 60-70 years in all the analyzed periods (Figure 2).

Age distribution in men demonstrated the first peak around age 56 to 65 and the second around age 41 to 45. The first peak was dominant until 2019, whereas the second peak appeared in 2018 and was higher in 2020 and 2021. The patient's body mass index (BMI) was comparable irrespective of the analyzed time interval (Table 1). The prevalence of comorbidities initially decreased from 76.8% in 2015-2016 to 69.2% in 2019, but increased again in 2020 and 2021 (70.9% and 73.9%, respectively). The proportion of the most frequent comorbidities of hypertension and diabetes ranged from 36.2% in 2020 to 48.7% in 2018 and from 17.4% in 2019 to 27% in 2021, respectively. The rate of patients using concomitant medications decreased from 76.8% in 2017 to 69.3% in 2020, with a slight increase to 71.8% in 2021.

Characteristics of HCV infection and liver disease severity

GT1b was the most common in all periods, but a reduction in the prevalence of infections with this GT from 89.2% to 62.8% between 2015 and 2020 was documented (Table 2). A renewed increase in the share of GT1b infection to 72.4% was noted in the last time frame analyzed. In parallel with the decrease in the percentage of GT1b, an increase in the share of GT3 was observed, ranging from 4.7% in 2015-2016 to 30.2% in 2020. Analogically to GT1b, a reversal of the earlier trend was documented in the last period. The percentage of patients classified as CP classes B and C, which was 10.5% in the first period analyzed, initially showed a downward trend, eventually increasing to 15.2% in the last time interval. No tendency was observed in the incidence of a history of hepatic decompensation, HCC, HBV, and HIV coinfection, as well as the presence of decompensation at baseline. Documented esophageal varices and a history of liver transplantation were more commonly observed at the beginning of the IFN-free era.

Treatment characteristics

There has been a strong and steady trend of an increasing share of treatment-naïve patients over the years (Table 3). At the beginning of the IFN-free era, the majority of those with previous treatment failure had been previously treated with PegIFN ± ribavirin (RBV), and this tendency was observed until 2018. Since 2019, two-thirds of individuals were retreated after the failure of IFN-free treatment. From 2015 to 2018, the most common therapeutic options were GT-specific regimens, particularly ombitasvir (OBV)/paritaprevir (PTV)/[ritonavir (r) ± dasabuvir (DSV) ± RBV] in 2015-2017 and grazoprevir (GZR)/[elbasvir (EBR) ± RBV] in 2018. In subsequent years, these regimens were replaced by pangenotypic options, which were used to treat about 90% of patients as of 2020. In 2019 and 2020, the glecaprevir (GLE)/pibrentasvir (PIB) combination was the most commonly used regimen, while in the last interval, most patients were treated with sofosbuvir (SOF)/[velpatasvir (VEL) ± RBV].

Treatment effectiveness

The overall SVR after excluding lost-to-follow-up patients was 95% (Table 4). A slightly higher effectiveness of 97.8% was noted in patients treated with the most common option of OBV/PTV/(r ± DSV ± RBV). A similar, high virologic response rate was achieved with other GT-specific regimens [LDV/(SOF ± RBV), GZR/(EBR ± RBV), and pangenotypic GLE/PIB combinations]. The administration of another pangenotypic option, VEL/(SOF ± RBV), resulted in a 91.6% cure rate. The lowest SVR rates were observed in those treated with SOF + RBV and asunaprevir (ASV) + daclatasvir (DCV) regimens - 72.9% and 85.5%, respectively. The smallest subgroups of patients treated with GLE/PIB/(SOF + RBV), SOF + (DCV ± RBV), SOF + (simeprevir ± RBV), and voxilaprevir (VOX)/VEL/SOF achieved a 100% treatment response.

Treatment effectiveness remained nearly similar in all six analyzed time intervals (Figure 3A). Analysis of SVR by GT showed comparable rates for all periods analyzed. The lowest response rates were obtained in GT3-infected patients (Figure 3C). A statistically significant higher SVR was observed in women between the first period analyzed and 2018 (Figure 3B). Univariate analysis showed that patients who did not achieve a virological response were significantly older, were more likely to be male, treatment-experienced, and had a higher BMI (Table 5).

The non-responding patient population also had a higher percentage of patients with GT3 infection, decompensated liver disease (CP classes B and C), and the presence of esophageal varices and liver decompensation in the form of encephalopathy at baseline. Those not responding virologically to treatment were significantly more likely to receive the ASV + DCV and SOF/(VEL ± RBV) regimens. A statistically significant difference in platelet count, bilirubin, and albumin levels between responders and virologic non-responders was also documented. Independent negative predictors of SVR in the

Table 1 Baseline characteristics of 3577 patients with cirrhosis treated with interferon-free regimens

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
Gender, females/males, *n* (%)	600 (50)/599 (50)	401 (48.5)/426 (51.5)	251 (43.8)/322 (56.2)	164 (37.4)/274 (62.6)	71 (35.7)/128 (64.3)	140 (41.1)/201 (58.9)
Age (yr), mean ± SD; min-max	58.6 ± 11.8; 21-97	59.4 ± 12.0; 22-87	58.2 ± 13.8; 21-89	55.2 ± 13.2; 26-91	54.6 ± 12.1; 28-83	56.6 ± 12.6; 25-86
Females	62.2 ± 10.8; 25-91	62.1 ± 11; 25-85	61.8 ± 14.2; 21-89	60.3 ± 12.8; 30-91	59.4 ± 12.2; 29-83	60.5 ± 12.3; 25-85
Males	55.1 ± 11.8; 21-97	56.8 ± 12.3; 22-87	55.4 ± 12.8; 27-88	52.1 ± 12.5; 26-89	51.9 ± 11.2; 28-80	53.9 ± 12.1; 30-86
Median (Q1, Q3)	60 (52, 67)	60 (52, 67)	59 (48, 68)	56 (45, 63)	55 (45, 63)	57 (47, 65)
Females, median (Q1, Q3)	63 (57, 69)	62 (56, 70)	62 (54, 73)	61 (52.5, 68)	60 (52, 68)	61 (53, 68.5)
Males, median (Q1, Q3)	57 (47, 63)	57 (48, 65)	56 (45, 64)	51 (42, 61)	52 (44, 60)	52 (44, 63)
BMI, mean ± SD; min-max	27.3 ± 4.5; 13.4-49.4	27.5 ± 4.8; 14.2-45.5	27.1 ± 4.7; 21-89	27.7 ± 5.2; 17-52.5	27.9 ± 5.6; 16-57.4	27.5 ± 4.9; 15.6-50
Comorbidities, *n* (%)						
Any comorbidity	921 (76.8)	682 (82.5)	431 (75.2)	303 (69.2)	141 (70.9)	252 (73.9)
Hypertension	582 (48.5)	384 (46.4)	279 (48.7)	179 (40.9)	72 (36.2)	158 (46.3)
Diabetes	263 (21.9)	212 (25.6)	120 (20.9)	76 (17.4)	44 (22.1)	136 (27)
Renal disease	48 (4)	39 (4.7)	20 (3.5)	10 (2.3)	9 (4.5)	12 (3.5)
Autoimmune diseases	29 (2.4)	14 (1.7)	8 (1.4)	9 (2.1)	2 (1)	3 (0.9)
Non-HCC tumors	20 (1.7)	15 (1.8)	12 (2.1)	12 (2.7)	6 (3)	15 (4.4)
Other	628 (52.4)	560 (67.7)	352 (61.4)	208 (47.5)	147 (73.9)	179 (52.5)
Concomitant medications, *n* (%)	879 (73.3)	635 (76.8)	431 (75.2)	305 (69.6)	138 (69.3)	245 (71.8)
ALT IU/L, mean ± SD	97.3 ± 66.2	96.4 ± 69.9	96 ± 85	107.1 ± 91.7	114.5 ± 103.8	115.9 ± 101.2
Bilirubin mg/dL, mean ± SD	1.2 ± 0.9	1 ± 0.7	1.1 ± 0.9	1.1 ± 0.9	1.1 ± 1.6	1.1 ± 0.9
Albumin g/dL, mean ± SD	5.8 ± 12.8	3.8 ± 0.5	3.8 ± 0.5	3.9 ± 0.5	3.8 ± 0.5	3.9 ± 0.6
Creatinine mg/dL, mean ± SD	1.1 ± 3.8	0.8 ± 0.5	0.9 ± 0.5	0.9 ± 0.7	0.9 ± 0.3	0.9 ± 0.7
Hemoglobin g/dL, mean ± SD	14.1 ± 1.8	14.1 ± 1.8	14 ± 1.8	14.1 ± 1.9	14.2 ± 1.8	13.9 ± 1.9
Platelets, × 1000/μL, mean ± SD	118.7 ± 61.6	136.1 ± 67.9	135 ± 70	136.5 ± 61.9	145.8 ± 87.6	145.6 ± 76.9
HCV RNA × 10^6 IU/mL, mean ± SD	1.6 ± 4.6	2.5 ± 6.4	2 ± 4.8	3.3 ± 24	1.7 ± 2.6	2 ± 3.2

IFN: Interferon; SD: Standard deviation; BMI: Body mass index; HCC: Hepatocellular carcinoma; ALT: Alanine transaminase; HCV RNA: Ribonucleic acid of hepatitis C virus.

logistic regression analysis were male gender [odds ratio (OR) = 2.28], GT3 infection (OR = 6.4), and prior failed treatment (OR = 1.66), while OBV/PTV/(r ± DSV ± RBV) therapy significantly increased the chance of response (OR = 0.58) (Table 6).

Treatment safety

As shown in Table 7, increasing tolerability was observed over time. The higher percentage of patients who completed a course of therapy as scheduled, from 89.5% (in 2015-2016) to 97.9% (in 2021), was assisted by a decreasing incidence of AEs, including serious ones, those leading to treatment discontinuation, and deaths. The most commonly reported AEs were mild, and weakness or fatigue remained the most frequent in all subsequent time intervals. The improvement in safety profile was accompanied by a decrease in RBV use from 71.3% in 2015-2016 to 6.7% in 2021. None of the 45 deaths were reported by the treating physician as related to antiviral therapy.

Table 2 Characteristics of liver disease in six-time intervals

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
GT, *n* (%)						
1	17 (1.4)	22 (2.7)	15 (2.6)	7 (1.6)	1 (0.5)	7 (2.1)
1a	21 (1.8)	17 (2.1)	10 (1.7)	10 (2.3)	9 (4.5)	8 (2.3)
1b	1070 (89.2)	652 (78.8)	420 (73.3)	291 (66.4)	125 (62.8)	247 (72.4)
2	0	0	1 (0.2)	3 (0.7)	0	3 (0.9)
3	56 (4.7)	103 (12.4)	109 (19.0)	111 (25.3)	60 (30.2)	63 (18.5)
4	35 (2.9)	33 (4.0)	17 (3)	16 (3.7)	4 (2)	13 (3.8)
5	0	0	0	0	0	0
6	0	0	1 (0.2)	0	0	0
Child-Pugh class, *n* (%)						
A	1033 (86.2)	724 (87.6)	521 (90.9)	397 (90.7)	176 (88.4)	285 (83.6)
B	118 (9.8)	77 (9.3)	47 (8.2)	36 (8.2)	18 (9.1)	49 (14.3)
C	9 (0.7)	1 (0.1)	2 (0.4)	1 (0.2)	1 (0.5)	3 (0.9)
No data	39 (3.3)	25 (3.0)	3 (0.5)	4 (0.9)	4 (2.0)	4 (1.2)
MELD score, *n* (%)						
< 15	1072 (89.4)	757 (91.5)	529 (92.3)	414 (94.5)	188 (94.5)	319 (93.5)
15-18	45 (3.8)	28 (3.4)	29 (5.1)	12 (2.8)	3 (1.5)	14 (4.1)
19-20	11 (0.9)	12 (1.5)	5 (0.9)	4 (0.9)	2 (1)	4 (1.2)
> 20	13 (1.1)	4 (0.5)	7 (1.2)	3 (0.7)	2 (1)	2 (0.6)
No data	58 (4.8)	26 (3.1)	3 (0.5)	5 (1.1)	4 (2)	2 (0.6)
History of hepatic decompensation, *n* (%)						
Ascites	124 (10.3)	65 (7.8)	64 (11.2)	26 (5.9)	15 (7.5)	47 (13.8)
Encephalopathy	33 (2.8)	15 (1.8)	19 (3.3)	4 (0.9)	2 (1)	7 (2.1)
Documented esophageal varices, *n* (%)	375 (31.3)	202 (24.4)	127 (22.2)	85 (19.4)	38 (19.1)	58 (17)
Hepatic decompensation at baseline, *n* (%)						
Moderate ascites-responded to diuretics	47 (3.9)	30 (3.6)	29 (5.1)	22 (5)	10 (5)	29 (8.5)
Tense ascites-not responded to diuretics	3 (0.3)	0	2 (0.3)	0	0	3 (0.9)
Encephalopathy	30 (2.5)	13 (1.6)	7 (1.2)	7 (1.6)	2 (1)	9 (2.6)
HCC history, *n* (%)	57 (4.8)	30 (3.6)	19 (3.3)	15 (3.4)	7 (3.5)	19 (5.6)
OLTx history, *n* (%)	23 (1.9)	4 (0.5)	1 (0.2)	1 (0.2)	0	1 (0.3)
HBV coinfection (HBsAg+), *n* (%)	12 (1)	12 (1.5)	12 (2.1)	8 (1.8)	3 (1.5)	6 (1.8)
HIV coinfection, *n* (%)	13 (1.1)	16 (1.9)	16 (2.8)	28 (6.4)	9 (4.5)	10 (2.9)

GT: Genotype; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; OLTx: Orthotopic liver transplantation; HBV: Hepatitis B virus; HBsAg+: Hepatitis B surface antigen; HIV: Human immunodeficiency virus.

DISCUSSION

The WHO has established the goal of eliminating HCV as a significant public threat by 2030, defined as a 90% reduction in new chronic infections and a 65% reduction in mortality compared to 2015[1]. In Poland, in middle-2015, the introduction of a therapeutic regimen program of IFN-free regimens for patients with CHC gave hope that the WHO goal could be achieved in our country. The key to reducing

Table 3 Treatment characteristics in six-time intervals

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
History of previous therapy						
Treatment-naïve	543 (45.3)	532 (64.3)	470 (82.0)	385 (87.9)	171 (86)	318 (93.2)
Nonresponder	290 (24.2)	103 (12.5)	23 (4.0)	7 (1.6)	6 (3)	11 (3.2)
Relapser	173 (14.4)	72 (8.7)	49 (8.5)	35 (8)	17 (8.5)	6 (1.8)
Discontinuation due to safety reason	71 (5.9)	40 (4.8)	14 (2.5)	4 (0.9)	1 (0.5)	3 (0.9)
Unknown type of response	120 (10.0)	76 (9.2)	14 (2.5)	7 (1.6)	2 (1)	3 (0.9)
No data	2 (0.2)	4 (0.5)	3 (0.5)	0	2 (1)	0
Number of patients with treatment failure	n = 654	n = 291	n = 100	n = 53	n = 26	n = 23
IFN ± RBV	64 (9.8)	36 (12.4)	2 (2)	0	0	0
PegIFN ± RBV	390 (59.6)	197 (67.7)	48 (48)	14 (26.4)	9 (34.6)	9 (39.1)
PegIFN + RBV + DAA	196 (30)	47 (16.1)	10 (10)	3 (5.7)	0	0
IFN-free	4 (0.6)	11 (3.8)	39 (39)	35 (66)	17 (65.4)	14 (60.9)
No data	0	0	1 (1)	1 (1.9)	0	0
Current treatment regimen						
Genotype-specific treatment regimens						
ASV + DCV	42 (3.5)	13 (1.6)	0	0	0	0
LDV/(SOF ± RBV)	350 (29.2)	283 (34.2)	154 (26.9)	16 (3.7)	5 (2.5)	4 (1.2)
OBV/PTV/(r ± DSV ± RBV)	745 (62.1)	327 (39.5)	32 (5.6)	0	0	1 (0.3)
GZR/(EBR ± RBV)	0	97 (11.7)	205 (35.8)	76 (17.3)	16 (8)	15 (4.4)
Other	62 (5.2)	104 (12.6)	19 (3.3)	0	0	0
SOF ± SMV ± DCV ± RBV, SMV ± DCV ± RBV						
Pangenotypic regimens						
GLE/PIB	0	1 (0.1)	59 (10.3)	178 (40.6)	99 (49.8)	117 (34.3)
GLE/(PIB + SOF + RBV)	0	0	0	2 (0.5)	0	0
SOF/(VEL ± RBV)	0	2 (0.3)	104 (18.1)	166 (37.9)	79 (39.7)	195 (57.2)
VOX/VEL/SOF	0	0	0	0	0	9 (2.6)
Current-RBV-containing therapies	855 (71.3)	360 (43.5)	163 (28.4)	44 (10)	12 (6)	23 (6.7)

IFN: Interferon; RBV: Ribavirin; PegIFN: Pegylated interferon; DAA: Direct-acting antivirals; ASV: Asunaprevir; DCV: Daclatasvir; LDV: Ledipasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; GZR: Grazoprevir; EBR: Elbasvir; SMV: Simeprevir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; VOX: Voxilaprevir.

mortality was using antiviral treatment in patients with the most advanced liver disease, as this population is most at risk of death[21]. It should be mentioned at this point that some countries initially reimbursed the therapy only for patients with advanced fibrosis and cirrhosis[22].

Despite the lack of restrictions in the Polish drug program, at the beginning of the IFN-free era, cirrhotic patients had priority in access to treatment due to long waiting lists. In the current study, they accounted for almost 44% of all treated patients in 2015-2016. This percentage decreased in consecutive periods, but the trend has been reversed since 2019. In the latter two intervals, not only were patients with cirrhosis reported more frequently, but this was also accompanied by an increase in the share of those with liver decompensation. One possible reason for the increase in cirrhosis rates is newly diagnosed cases in advanced stages of the disease in patients previously unaware of HCV infection[23]. This hypothesis is supported by the increasing percentage of patients previously untreated. At the same time, the total number of treated patients decreased. Other researchers point to the negative impact of

Table 4 Treatment effectiveness according to regimen, calculated as per protocol analysis

Regimen	SVR PP, *n* (%)
All regimens	3271/3442 (95)
OBV/PTV/(r ± DSV ± RBV)	1052/1076 (97.8)
LDV/(SOF ± RBV)	748/778 (96.1)
GZR/(EBR ± RBV)	387/398 (97.2)
VEL/(SOF ± RBV)	477/521 (91.6)
GLE/PIB	420/435 (96.6)
GLE/PIB/(SOF + RBV)	2/2 (100)
ASV + DCV	47/55 (85.5)
SOF + DCV ± RBV	21/21 (100)
SOF + RBV	105/144 (72.9)
SOF + SMV ± RBV	5/5 (100)
VOX/VEL/SOF	7/7 (100)

PP: Per protocol; SVR: Sustained virological response; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; RBV: Ribavirin; LDV: Ledipasvir; SOF: Sofosbuvir; GZR: Grazoprevir; EBR: Elbasvir; VEL: Velpatasvir; GLE: Glecaprevir; PIB: Pibrentasvir; ASV: Asunaprevir; DCV: Daclatasvir; SMV: Simeprevir; VOX: Voxilaprevir.

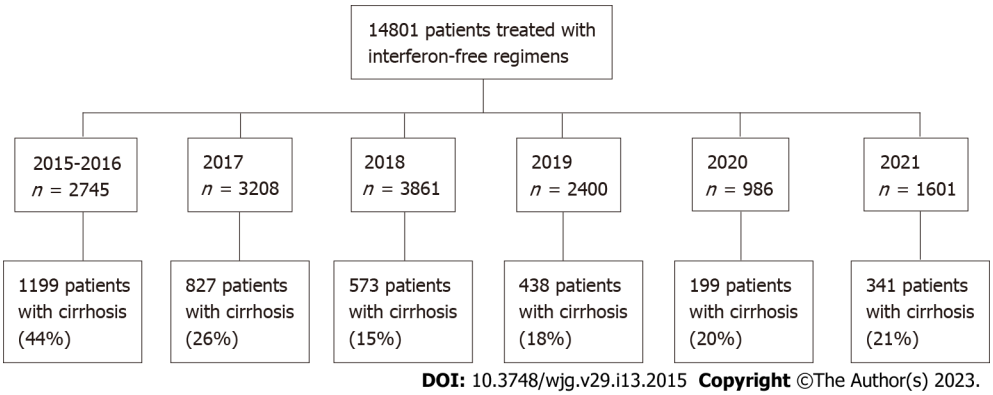

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Figure 1 Distribution of patients treated for hepatitis C virus infection in six-time intervals.

the coronavirus disease 2019 (COVID-19) pandemic on the number of diagnosed and treated patients with chronic HCV infection[24,25].

To the best of our knowledge, this is the first study to track changes in patient profiles, therapeutic options used, and their efficacy in patients with cirrhosis in the IFN-free era. Our work summarizes 7 years of access to IFN-free therapy in Poland. In the current study, a slight decrease in the age of patients with cirrhosis was observed between the first and last time interval. This reduction was much less pronounced compared to analyzes of demographic changes in CHC patients regardless of liver fibrosis[11,13]. The differences can be explained by the fact that the cirrhotic patient population tended to be older compared to patients with non-advanced liver fibrosis. The age distribution was irregular and evolved throughout the entire analyzed period. Although the two-peak pattern was described previously not only in the Polish population with CHC, we noted only one peak at ages 60 to 70 in women in all six-time intervals. We observed a second peak around the age of 41 to 45, only in men in 2018-2021[11,13,26].

In addition to prioritizing cirrhotic patients at the beginning of the IFN-free era, those after previous treatment failure were also given priority in access to therapy. This explains the decreasing percentage of treatment-experienced patients over time, and it should be emphasized that despite the COVID-19 pandemic in 2020 and 2021, this trend continued. Patients after previous ineffective antiviral treatment up to 2018 were more likely to be on failed IFN-based regimens, while 2019-2021 documented a higher proportion of patients on failed DAA therapy, which is understandable, given the evolution and availability of antiviral regimens. An additional factor contributing to the growing proportion of treatment-naïve individuals was the detection of HCV infection in patients previously unaware of it.

Table 5 Comparison of responders and virological non-responders to antiviral therapy

Parameter	Responders (*n* = 3271)	Non-responders (*n* = 171)	*P* value
Gender, females/males, *n* (%)	1539 (47)/1732 (53)	42 (24.6)/129 (75.4)	< 0.0001
Age (yr), mean ± SD; min-max	58.0 ± 12.6; 21-97	55.0 ± 10.5; 29-84	0.0002
Females	61.6 ± 11.8; 21-91	59.9 ± 10.6; 35-81	0.3173
Males	54.8 ± 12.4; 21-97	53.4 ± 10; 29-84	0.1045
BMI, mean ± SD; min-max	27.4 ± 5.0; 13.4-57.4	28.7 ± 4.6; 16-47.5	0.0001
Current treatment regimen			
Genotype-specific treatment regimens			
ASV + DCV	47 (1.4)	8 (4.7)	0.0001
LDV/(SOF ± RBV)	748 (22.9)	30 (17.5)	0.1047
OBV/PTV/(r ± DSV ± RBV)	1052 (32.2)	24 (14)	< 0.0001
GZR/(EBR ± RBV)	387 (11.8)	11 (6.4)	0.0314
Pangenotypic regimens			
GLE/PIB	420 (12.8)	15 (8.8)	0.1186
GLE/(PIB + SOF + RBV)	2 (0.1)	0	0.7464
SOF/(VEL ± RBV)	477 (14.6)	44 (25.7)	0.0001
VOX/VEL/SOF	7 (0.2)	0	0.5448
GT, *n* (%)			< 0.0001
1	63 (1.9)	2 (1.2)	
1a	70 (2.2)	1 (0.6)	
1b	2624 (80.2)	89 (52.0)	
3	397 (12.1)	76 (44.4)	
4	110 (3.4)	3 (1.8)	
Other	7 (0.2)	0	
Comorbidities, *n* (%)			
Any comorbidity	2498 (76.4)	131 (4)	0.9425
Hypertension	1533 (46.9)	66 (2)	0.0345
Diabetes	731 (22.3)	46 (1.4)	0.1651
Renal disease	124 (3.8)	8 (0.2)	0.5558
Autoimmune diseases	63 (1.9)	0	0.0670
Non-HCC tumors	68 (2.1)	6 (0.2)	0.1963
Other	1783 (54.5)	96 (2.9)	0.6762
Concomitant medications, *n* (%)	2389 (73.0)	136 (79.5)	0.0610
Treatment experienced	1039 (31.8)	69 (40.4)	0.0208
History of hepatic decompensation, *n* (%)			
Ascites	297 (9.0)	21 (12.2)	0.1588
Encephalopathy	65 (2.0)	7 (4.1)	0.0606
Documented esophageal varices, *n* (%)	780 (23.8)	64 (37.4)	0.0001
Hepatic decompensation at baseline, *n* (%)	173 (5.3)	18 (10.5)	0.0035
HCC history, *n* (%)	117 (3.6)	9 (5.3)	0.2523
OLTx history, *n* (%)	28 (0.9)	1 (0.6)	0.7052
Child-Pugh class, *n* (%)			

B or C	296 (9)	29 (17)	0.0006
HBV coinfection (HBsAg+), *n* (%)	50 (1.5)	2 (1.2)	0.7075
HIV coinfection, *n* (%)	79 (2.4)	6 (3.5)	0.3690
ALT IU/L, mean ± SD	101.2 ± 80.8	106.9 ± 82.0	0.3385
Bilirubin mg/dL, mean ± SD	1.1 ± 0.9	1.2 ± 0.7	< 0.0001
Albumin g/dL, mean ± SD	4.5 ± 7.7	3.9 ± 3.1	0.0002
Creatinine mg/dL, mean ± SD	0.9 ± 2.3	0.8 ± 0.2	0.8562
Hemoglobin g/dL, mean ± SD	14.1 ± 1.8	13.9 ± 1.8	0.1319
Platelets, × 1000/μL, mean ± SD	132.6 ± 66.8	109.2 ± 59.4	< 0.0001
HCV RNA × 10^6 IU/ml, mean ± SD	2.2 ± 10.0	2.0 ± 2.9	0.1123

SD: Standard deviation; BMI: Body mass index; ASV: Asunaprevir; DCV: Daclatasvir; LDV: Ledipasvir; SOF: Sofosbuvir; RBV: Ribavirin; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir; GZR: Grazoprevir; EBR: Elbasvir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; VOX: Voxilaprevir; GT: Genotype; HCC: Hepatocellular carcinoma; OLTx: Orthotopic liver transplantation; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HIV: Human immunodeficiency virus; ALT: Alanine transaminase; HCV RNA: Ribonucleic acid of hepatitis C virus.

Table 6 Logistic multiple regression results on association between selected parameters significant in univariate analysis and non-response to antiviral treatment

Parameter	OR	95%CI	*P* value
Age ≥ 75 yr	0.81	0.38-1.72	0.5915
Obesity (BMI ≥ 30 kg/m^2)	1.15	0.80-1.66	0.4473
Male gender	2.28	1.57-3.29	< 0.0001
Platelets < 100000/μL	1.37	0.98-1.93	0.0694
GT1b	1.54	0.66-3.59	0.3145
GT3	6.40	2.69-15.22	< 0.0001
Bilirubin > 3 mg/dL	0.29	0.08-1.00	0.0506
Albumin < 2.8 g/dL	0.98	0.44-2.20	0.966
Documented esophageal varices	1.43	1.00-2.06	0.052
Hepatic decompensation at baseline	1.49	0.76-2.91	0.2496
Hypertension	0.93	0.67-1.30	0.6783
Child-Pugh, B or C	1.80	0.95-3.42	0.0716
SOF/(VEL ± RBV)	1.13	0.76-1.69	0.541
GZR/(EBR ± RBV)	0.91	0.46-1.80	0.7951
OBV/PTV/(r ± DSV ± RBV)	0.58	0.35-0.95	0.0319
Treatment experienced	1.66	1.18-2.33	0.0039

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; GT: Genotype; SOF: Sofosbuvir; VEL: Velpatasvir; RBV: Ribavirin; GZR: Grazoprevir; EBR: Elbasvir; OBV: Ombitasvir; PTV/r: Paritaprevir; DSV: Dasabuvir.

Since DAA therapy lasts much shorter than previously used IFN-based regimens, in the absence of restrictions on treatment reimbursement, the waiting list was shortened considerably and subsequently ceased to exist, while antiviral therapy is implemented immediately after the diagnosis is established [27]. This makes the characteristics of newly diagnosed patients quickly change the profile of the patients treated.

There was also a significant change in the share of HCV GTs in the treated patients. The most common HCV GT in Poland was GT1b[28,29]. Our study restricted to individuals with cirrhosis confirmed these findings, but we noted a changing distribution of GTs over time depending on the availability of treatment regimens in subsequent years. At the beginning of the IFN-free era, GT1- and GT4-infected patients had access to highly effective GT-specific regimens, while patients with GT3

Table 7 Safety of antiviral therapy in six-time intervals

Parameter	2015-2016	2017	2018	2019	2020	2021
Number of patients	1199	827	573	438	199	341
Treatment course, *n* (%)						
According to schedule	1073 (89.5)	776 (93.8)	538 (93.9)	428 (97.7)	196 (98.5)	334 (97.9)
Therapy modification	71 (5.9)	21 (2.6)	26 (4.5)	2 (0.5)	0	0
Therapy discontinuation	44 (3.7)	19 (2.3)	9 (1.6)	5 (1.1)	1 (0.5)	6 (1.8)
No data	11 (0.9)	11 (1.3)	0	3 (0.7)	2 (1.0)	1 (0.3)
Patients with at least one AE, *n* (%)	500 (41.7)	212 (25.6)	139 (24.3)	80 (18.3)	36 (18.1)	66 (19.4)
Serious adverse events, *n* (%)	48 (4)	11 (1.3)	16 (2.8)	7 (1.6)	4 (2)	3 (0.9)
AEs leading to treatment discontinuation, *n* (%)	35 (2.9)	16 (1.9)	4 (0.7)	2 (0.5)	1 (0.5)	1 (0.3)
Most common AEs (≥ 2%), *n* (%)						
Weakness/fatigue	242 (20.2)	78 (9.4)	59 (10.3)	28 (6.4)	13 (6.5)	16 (4.7)
Anemia	63 (5.3)	36 (4.4)	17 (3)	2 (0.5)	0	2 (0.6)
Sleep disorders	52 (4.3)	20 (2.4)	20 (3.5)	4 (0.9)	6 (3)	6 (1.8)
Nausea	32 (2.7)	14 (1.7)	3 (0.5)	1 (0.2)	5 (2.5)	6 (1.8)
Abdominal pain	21 (1.8)	7 (0.8)	4 (0.7)	12 (2.7)	5 (2.5)	8 (2.3)
Headaches	50 (4.2)	14 (1.7)	11 (1.9)	7 (1.2)	3 (0.5)	8 (2.3)
Myalgia/arthralgia	18 (1.5)	18 (2.2)	9 (1.6)	6 (1.4)	6 (3)	8 (2.3)
Pruritis	71 (5.9)	19 (2.3)	10 (1.7)	10 (2.3)	7 (3.5)	7 (2.1)
Skin lesions	26 (2.2)	14 (1.7)	7 (1.2)	5 (1.1)	2 (1)	4 (1.2)
Bilirubin, > ULN	44 (3.7)	30 (3.6)	3 (0.5)	3 (0.7)	0	0
AEs of particular interest						
Ascites	30 (2.5)	23 (2.8)	12 (2.1)	6 (1.4)	0	13 (3.8)
Hepatic encephalopathy	28 (2.3)	12 (1.5)	6 (1.0)	2 (0.5)	1 (0.5)	6 (1.8)
Gastrointestinal bleeding	9 (0.8)	4 (0.5)	5 (0.9)	1 (0.2)	1 (0.5)	0
Death, *n* (%)	13 (1.1)	10 (1.2)	10 (1.7)	3 (0.7)	2 (1.0)	7 (2.1)

AE: Adverse event; ULN: Upper limit of normal.

infection in Poland were still treated with IFN-based options as the available SOF + RBV was considered suboptimal due to lower SVR[30,31]. The SOF + (DCV ± RBV) regimen with activity against GT3 was used incidentally in Poland due to problems with reimbursement. However, our analysis confirmed the high effectiveness of this option. For this reason, patients infected with GT, especially subtype 1b, definitely dominated in the initially analyzed time intervals. In 2018, new pangenotypic options, including GLE/PIB and SOF/(VEL ± RBV), which are highly effective for all HCV GTs, became available in Poland, thus enabling effective treatment of patients infected with GT3. This was reflected in the increase in the percentage of this population, despite the still visible dominance of GT1b resulting from the original distribution of GTs in the Polish population. Our findings are also supported by an RWE study conducted in Germany among patients with HCV infection, regardless of cirrhosis status treated with DAA[32].

Surprisingly, in the last analyzed period, we again recorded an increase in GT1b infections accompanied by a decrease in the percentage of GT3-infected patients. It seems that this change could have been influenced by both effective therapies of patients with GT3 infection, as well as the previously described phenomenon of detecting HCV infections in previously undiagnosed people, who, according to the original distribution of HCV GTs in the Polish population, are more likely to be infected with GT1b.

The change in the profile of patients with cirrhosis treated with DAA regimens in Poland was accompanied by changes in the options used related to the registration of new drugs and recommendations of the Polish Group of Experts for HCV[15-19]. At the beginning of the IFN-free era, GT-specific regimens were used, especially the OBV/PTV/(r + DSV ± RBV) combination, which was the first

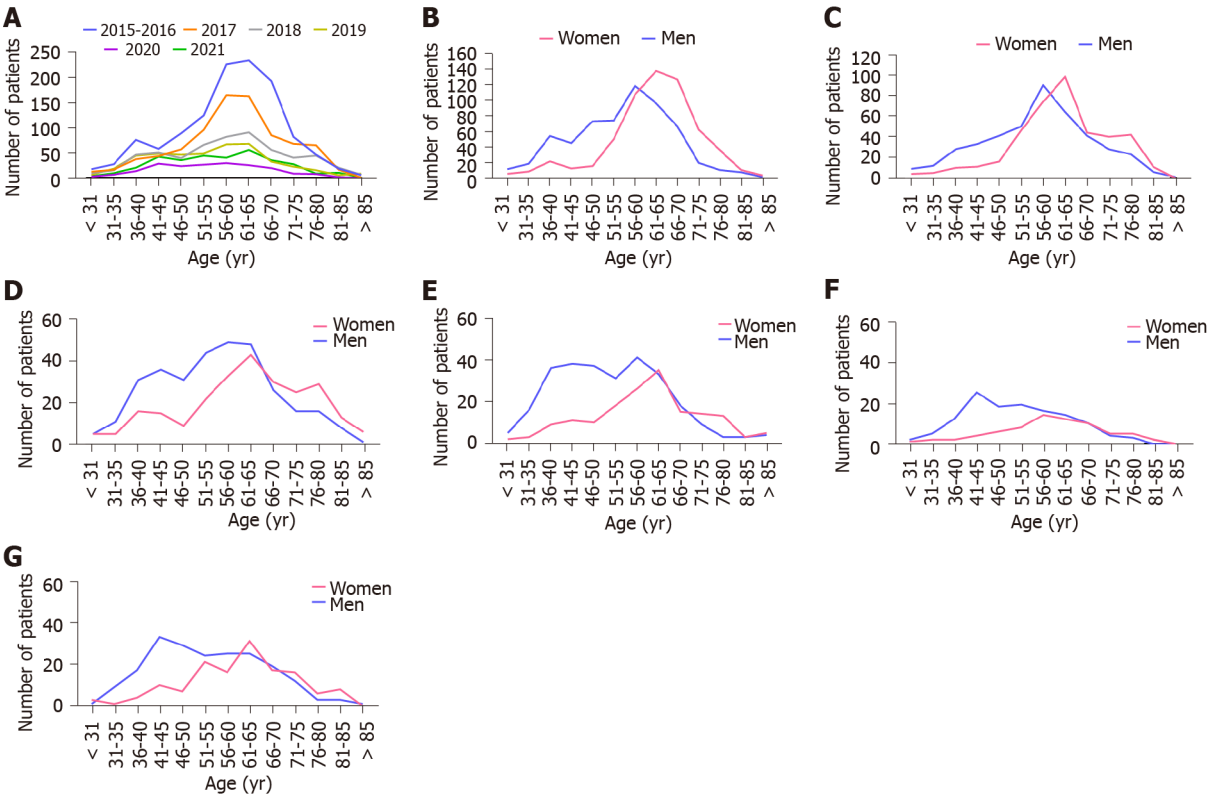

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Figure 2 Age distribution in all patients treated in six-time intervals and according to gender in 2015-2016, 2017, 2018, 2019, 2020, and 2021. A: In six-time intervals; B: In 2015-2016; C: In 2017; D: In 2018; E: In 2019; F: In 2020; G: In 2021.

reimbursed IFN-free therapy in Poland. As mentioned above, as of 2018, pangenotypic regimens have become available in our country, and this has gradually displaced previous therapeutic options.

Regardless of the changing patient profile and therapeutic options, the effectiveness of therapy in this difficult-to-treat group of patients with advanced liver disease remained consistently high in all analyzed periods. The effectiveness of the administrated regimens was very high and exceeded 95% for most options. We documented the highest SVR for the regimen OBV/PTV/(r + DSV ± RBV), which was found to be an independent success factor in the multivariate analysis (OR = 0.58). This was an option used in the largest number of patients in the current study. Its efficacy of 99% in the population of patients with advanced liver disease was previously documented in the AMBER RWE study[33]. The high effectiveness achieved after treatment with other GT-specific regimens is also consistent with the results of the RWE GZR/(EBR ± RBV) and LDV/(SOF ± RBV) studies[34-36].

An excellent response rate of 97.6% was achieved in the GLE/PIB group, and these results were supported by a meta-analysis of 18 RWE studies[37]. Another pangenotypic regimen of VEL/(SOF ± RBV) was effective in 91.6%. Although the large meta-analysis from 12 cohorts documented a 97.9% SVR in 1078 patients with cirrhosis, it should be noted that only compensated patients were included in that analysis. In our cohort, 14% of patients receiving this option were decompensated, which may have affected the results obtained[38]. The lowest SVR rate of 72.9% was achieved by patients treated with SOF + RBV. This suboptimal therapy was received by individuals with GT3 infection who had contraindications or intolerance to PegIFN. Our results are lower than those demonstrated in the BOSTON study, where 24-wk SOF + RBV therapy in patients with GT3 cirrhosis was 79% effective[30]. On the other hand, the HCV-TARGET real-world study documented the effectiveness of this regimen in only 45% of patients with cirrhosis and GT3 infection[39].

GT3 infection was found in our study as an independent predictor of reduced SVR rate in multivariate analysis, in addition to male gender and history of prior therapy. Our observations are consistent with the results of a large retrospective RWE study involving 15720 United States veterans with a 40% share of cirrhotics, in whom GT3 infection significantly reduced the chances of responding to DAA[40]. However, it should be noted that in the above-cited study, SOF + RBV + PegIFN, LDV/(SOF + RBV) options were used in patients with GT3 infection, and only the introduction of highly effective pangenotypic options improved the response rate in this population[41].

Treatment experience has been documented as a factor associated with DAA therapy failure in the RWE study conducted by Chen *et al*[42]. In contrast, Berkan-Kawińska *et al*[43] did not find that it was a negative predictor of SVR among patients with cirrhosis. A specific subgroup of treatment-experienced patients is the population after the previous failure of DAA regimens. Despite other reports indicating a

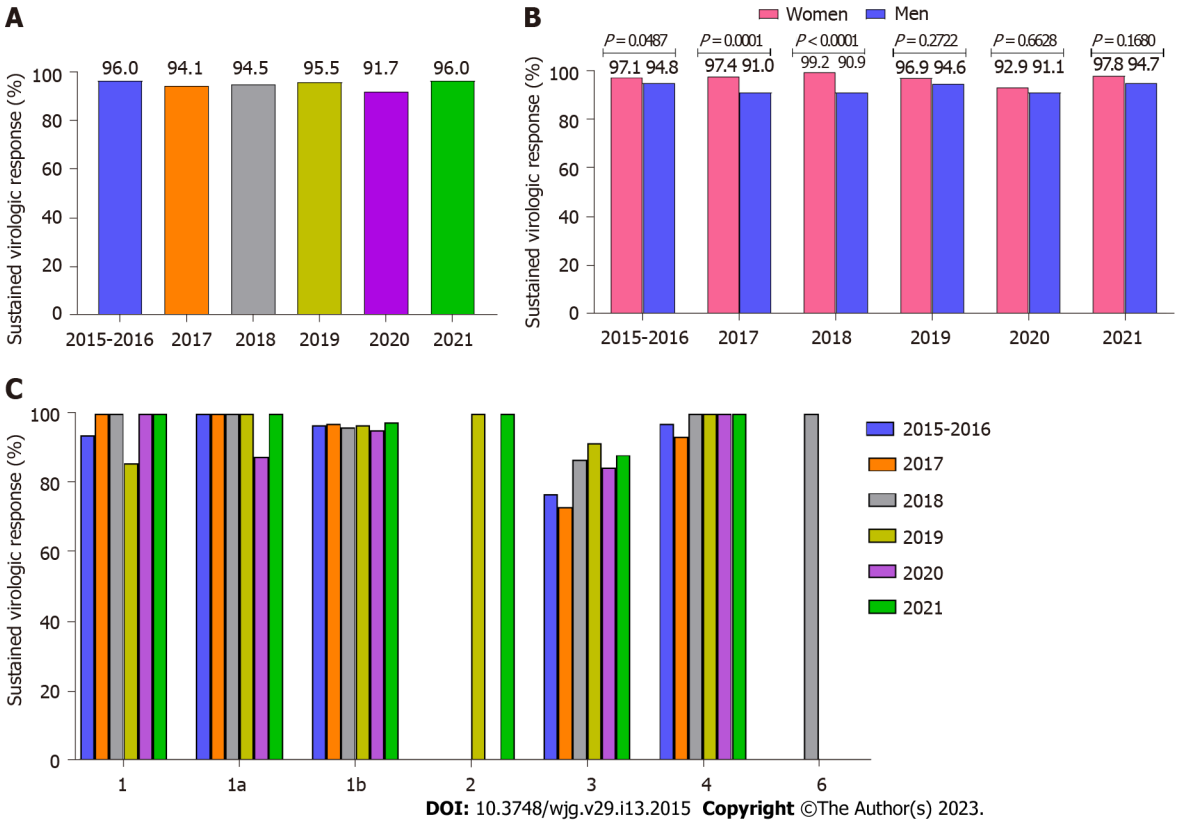

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Figure 3 Sustained virological response rates by hepatitis C virus genotype, in six-time intervals (per protocol analysis), and according to gender. A: In six-time intervals (per protocol analysis); B: According to gender; C: By hepatitis C virus genotype.

lower cure rate in DAA-experienced cirrhotic patients, the POLARIS-1 and POLARIS-4 clinical trials showed that these patients retreated with the pangenotypic SOF/VEL/VOX combination achieved high efficacy of 93%-98%[44-46]. Although in the current analysis, the pangenotypic rescue option of SOF/VEL/VOX or GLE/(PIB + SOF + RBV) was used in only 11 patients after failure of therapy with DAA regimens, all achieved a virological response.

Similar to our results, male gender was an independent negative predictor of achieving SVR in a large study from the United States Veterans Affairs[40]. Data from other RWE studies confirmed these results [34,47]. The high effectiveness of DAA regimens in our analysis was accompanied by a good safety profile with a low rate of discontinuation due to AEs, which supports findings from clinical trials and other RWE studies[34,40,48-50]. Importantly, we observed an improvement in the safety profile over time, which is explained by shortening the therapy and reducing the frequency of using regimens containing RBV. Weakness/fatigue was the most common AE regardless of the analyzed period. The percentage of patients with symptoms of liver decompensation during therapy and the 12-wk follow-up period did not exceed a few percent, which proves the good safety profile of DAA in patients with cirrhosis and is consistent with the observations of other authors[51]. This is also confirmed by the overall death rate of 1.3%, varying from 0.7% to 2.1% over time.

Limitations

Our analysis has several limitations typical of retrospective studies, including possible physician bias due to incomplete data, inconsistent diagnosis or misclassification of data, underreporting of AEs, and possible data entry errors. In addition, the observational nature of the study may result in insufficient discipline during treatment; we did not capture data on adherence to the treatment, while available data suggest that lack of adherence to therapy may result in a lower chance of virologic response in cirrhotic patients treated with the SOF/LDV regimen[52,53].

Although the study was conducted in the same centers treating patients, and it seems that the decreasing overall number of patients and the changing percentage of patients with cirrhosis reflect the actual condition, distortion of the data by other factors cannot be excluded. One of these may be that the analysis also covered the COVID-19 pandemic period, when the availability of diagnostic tests and antiviral therapy was limited.

However, it is important to mention the main strength of our study, which is the collection of data from a truly geographically diverse population, representative of routine practice and a large number of patients enrolled from different centers in our country. This allows the generalization of the results. Another strong point is the large number of patients retained in post-treatment evaluation, with a rather

low rate of those lost to follow-up (2.5% after the exclusion of deaths). Notably, the current analysis is the first study to directly compare changes in patient profile, antiviral treatment characteristics, efficacy, and treatment safety in patients with cirrhosis over time in the era of IFN-free therapy.

CONCLUSION

The current analysis documents changes in the characteristics of patients with HCV-infected cirrhosis that have occurred over the seven years of access to IFN-free therapies in the form of a decline in the median age of patients, the prevalence of comorbidities, and the use of concomitant medications. In addition, the evolution of the therapeutic regimens used is described, in the course of which GT-specific options were supplanted by pangenotypic regimens. Regardless of these changes, patients achieved consistently high efficacy in all analyzed periods. Male gender, GT3 infection, and prior treatment were identified as independent negative predictors of SVR.

ARTICLE HIGHLIGHTS

Research background

Direct-acting antivirals (DAAs), which have replaced interferon (IFN)-based regimens, have significantly improved the prognosis of patients with cirrhosis, the population at highest risk for the most severe complications of infection with hepatitis C virus (HCV).

Research motivation

We aimed to track changes in the characteristics of HCV-infected patients with cirrhosis and document the evolving treatment regimens over the years, along with their efficacy and safety profile in this patient population.

Research objectives

Data of 3577 cirrhotics selected from 14801 HCV-infected patients treated between 2015 and 2021 with DAA regimens derived from the Epiter-2 database were analyzed.

Research methods

The analysis used demographic, clinical, and laboratory data of the studied population collected retrospectively in the Epiter-2 database. The measure of treatment effectiveness was the percentage of sustained virologic response (SVR) calculated after excluding patients lost to follow-up. Safety data collected during therapy and the 12-wk post-treatment period included information on adverse events, including serious ones, deaths, and treatment course.

Research results

From 2015 to 2017, the study population was gender-balanced, while male dominance was evident in subsequent years. The decrease in the median age of patients documented during the study was accompanied by a decrease in the percentage of patients with comorbidities and comedications. A steady increase in the percentage of treatment-naïve patients was observed over the years. The genotype (GT)-specific options dominant in 2015-2018 were then replaced by pangenotypic regimens. The effectiveness of the therapy was comparable regardless of the period analyzed, and patients achieved an overall response rate of 95%, with an SVR range of 72.9%-100% for the different therapeutic regimens. Male gender, GT3 infection, and prior treatment failure were identified as independent negative predictors of therapeutic success.

Research conclusions

The study documents changes in the profile of HCV-infected cirrhotic patients over the years of accessibility to changing DAA regimens, confirming the high effectiveness of IFN-free therapy in all analyzed periods.

Research perspectives

Changes in the characteristics of patients, especially those with cirrhosis, may affect the expected change in the number of patients with liver cancer or at risk of decompensation. This knowledge is important from the point of view of planning the directions of health care development.

FOOTNOTES

Author contributions: Brzdęk M, Zarębska-Michaluk D, and Flisiak R conceived the study design, and analyzed and interpreted the data; Brzdęk M and Flisiak R prepared the figures; Brzdęk M prepared the tables; Brzdęk M, Zarębska-Michaluk D, and Flisiak R drafted the manuscript; Rzymski P performed the statistical analysis; Lorenc B, Kazek A, Tudrujek-Zdunek M, Janocha-Litwin J, Mazur W, Dybowska D, Berak H, Parfieniuk-Kowerda A, Klapaczyński J, Sitko M, Sobala-Szczygieł B, and Piekarska A acquired the data; Brzdęk M, Zarębska-Michaluk D, Rzymski P, and Flisiak R prepared a revised version of the manuscript; Brzdęk M and Zarębska-Michaluk D prepared the manuscript for the submission; and all authors approved the final version of the manuscript.

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Exploring choices of early nutritional support for patients with sepsis based on changes in intestinal microecology

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Abstract

BACKGROUND

Sepsis exacerbates intestinal microecological disorders leading to poor prognosis. Proper modalities of nutritional support can improve nutrition, immunity, and intestinal microecology.

AIM

To identify the optimal modality of early nutritional support for patients with sepsis from the perspective of intestinal microecology.

METHODS

Thirty patients with sepsis admitted to the intensive care unit of the General Hospital of Ningxia Medical University, China, between 2019 and 2021 with indications for nutritional support, were randomly assigned to one of three different modalities of nutritional support for a total of 5 d: Total enteral nutrition

(TEN group), total parenteral nutrition (TPN group), and supplemental parenteral nutrition (SPN group). Blood and stool specimens were collected before and after nutritional support, and changes in gut microbiota, short-chain fatty acids (SCFAs), and immune and nutritional indicators were detected and compared among the three groups.

RESULTS

In comparison with before nutritional support, the three groups after nutritional support presented: (1) Differences in the gut bacteria (Enterococcus increased in the TEN group, Campylobacter decreased in the TPN group, and Dialister decreased in the SPN group; all $P < 0.05$); (2) different trends in SCFAs (the TEN group showed improvement except for Caproic acid, the TPN group showed improvement only for acetic and propionic acid, and the SPN group showed a decreasing trend); (3) significant improvement of the nutritional and immunological indicators in the TEN and SPN groups, while only immunoglobulin G improved in the TPN group (all $P < 0.05$); and (4) a significant correlation was found between the gut bacteria, SCFAs, and nutritional and immunological indicators (all $P < 0.05$).

CONCLUSION

TEN is recommended as the preferred mode of early nutritional support in sepsis based on clinical nutritional and immunological indicators, as well as changes in intestinal microecology.

Key Words: Sepsis; Nutritional support; Intestinal microecology; Short-chain fatty acids; Nutritional and immunological indicators; Total enteral nutrition; Total parenteral nutrition; Supplemental parenteral nutrition

Core Tip: Nutritional support is an important component of treatment for sepsis, and an appropriate modality of nutritional support can improve patient nutrition, immunity, and intestinal microecology. We applied different nutritional modalities for early and short-duration nutritional support in patients with sepsis and found differences in intestinal bacterial composition, short-chain fatty acids, and nutritional and immune indicators. We concluded that total enteral nutrition is a good modality of early nutritional support for sepsis. These findings provide a new perspective for optimizing nutritional support modalities in sepsis.

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INTRODUCTION

Sepsis is a life-threatening organ dysfunction resulting from a dysregulated response of the body to infection[1]. Sepsis affects nearly 50 million people each year worldwide and accounts for approximately 11 million deaths, corresponding to approximately 20% of all deaths worldwide[2]. The high morbidity and mortality rates associated with sepsis, as well as the difficulties with early detection and treatment, have become challenging healthcare issues.

Our previous study confirmed the presence of gut microbiota disorders in patients with sepsis[3], and the interaction of disease and gut microbiota disorders has been shown to lead to clinical deterioration and the development of multiple organ dysfunction (MODS)[4]. Therefore, the treatment of sepsis requires consideration of the range of its effects on gut microbiota, metabolism, nutrition, and immunity. Nutritional support is an important component of sepsis treatment; however, its focus is still limited to the amount of energy provided, the choice of energy supply routes, the placement of feeding tubes, and nitrogen balance. The clinical recommendations about the choice of nutritional support modalities are largely based on expert consensus, although the supporting evidence is of low level[5,6]. Nutritional support has a significant impact on intestinal microecology; however, the modalities of nutritional support in sepsis have not been evaluated from the perspective of intestinal microecology.

In this context, we used different modalities of nutritional support: Total enteral nutrition (TEN), total parenteral nutrition (TPN), and supplemental parenteral nutrition (SPN) to group patients with sepsis.

Moreover, we explored the choices of nutritional modalities for sepsis from the perspective of systemic immunity and nutritional indicators, the degree of their interrelationships, and the effects of the three nutritional modalities on the intestine microecology [including gut microbiota, short-chain fatty acid (SCFA) metabolism]. In this study, 16S rRNA gene sequencing and GC-MS metabolomics were used to detect gut microbiota and SCFAs. The exploration of early nutritional support in patients with sepsis from the perspective of intestinal microecology not only provides a new fundamental perspective for optimizing nutritional support in sepsis but also has far-reaching implications for improving nutritional support.

MATERIALS AND METHODS

General information

A randomized clinical trial involving 30 patients with sepsis admitted to the Department of Critical Care Medicine at the General Hospital of Ningxia Medical University (a Grade 3A hospital) between October 1, 2019, and February 1, 2021, was conducted. The inclusion criteria were as follows: (1) Age between 18 and 75 years; (2) meeting the criteria of the latest definition of sepsis of the American Society of Critical Care Medicine "Sepsis-3.0" from 2016[1]; (3) mechanically ventilated patients; and (4) indications for nutritional support [hemodynamic stability (no dose or small dose of vasoactive drugs to maintain vital signs and lactate ≤ 2 mmol/L) and NUTRIC score (Nutritional Risk Rating Instrument for Critically Ill Patients) ≥ 6]. The following exclusion criteria applied: (1) Patients with chest wall, thoracic, or airway injuries, respiratory instability, ventilator parameters requiring adjustment during measurement, or rapid respiratory rate ≥ 35 breaths/min, or oxygen concentration > 60%, or positive end-expiratory pressure ≥ 10 cmH_2O; (2) patients with open injuries to the gastrointestinal tract or abdominal cavity; (3) patients with severe acid-base balance or electrolyte metabolism disturbances; and (4) patients whose family members did not provide informed consent to participate in the trial. Informed consent was obtained from the patients or their immediate family members. This study was approved by the Ethics Committee of the Hospital (approval number: 2017-266).

Patients with sepsis included in this study were divided into three groups using a random number table and a random number remainder grouping method. Nutritional support was administered to the patients *via* the following different modalities based on the order of randomization: (1) TEN group (n = 10): Total enteral nutritional support *via* a gastric tube or jejunal tube feeding; (2) TPN group (n = 10): Total parenteral nutrition *via* the internal jugular vein; and (3) SPN group (n = 10): Trophic enteral nutrition *via* a gastric tube or jejunal tube feeding + SPN *via* the internal jugular vein, in which trophic enteral is 10–20 kcal/h, not more than 500 kcal in 24 h, and the remaining energy is supplemented by parenteral nutrition[6]. The amount of nutritional support provided to the patients in the three groups was based on the resting energy expenditure (REE) measured by indirect calorimetry in the ventilation circuit; the energy metabolism test (metabolic cart) used in this study was Vmax Encore 229, Medical Graphics, United States, 2015. The selected SPN enteral preparation for enteral nutrition solution was Enteral Nutritional Suspension (SP, 500 mL/500 kcal, Nutricia Pharmaceutical Co., Ltd.). The selected TEN enteral preparation for enteral nutrition solution, due to energy supply and fluid volume regulation, was Enteral Nutritional Emulsion (TPF, total energy 500 mL/750 kcal, Nutricia Pharmaceutical Co., Ltd.), combined or not with SP. Patients who received nutritional support for less than 5 d during the nutritional support period because of changes in patient condition (discharge from hospital after improvement in condition or inability to continue with nutritional support owing to deterioration in condition) were regarded as invalid observation cases and excluded from the study. Subsequently, the next randomized patient was administered nutritional support using the same modality used for the invalid case. Patient age, sex, underlying disease, NUTRIC score, Acute Physiology and Chronic Health Evaluation (APACHE II) score, sequential organ failure assessment (SOFA) score, and general information such as REE during 24 h, were recorded. Before day 1 and on day 5 of nutritional support, stool specimens were collected for 16S rRNA gene sequencing and the analysis of SCFA metabolism and venous blood specimens were collected for lymphocyte subpopulation analysis, immunoglobulin + complement analysis, and complete set of nutritional tests.

Fecal specimen collection and sequencing

Stool specimens were collected from enrolled patients with sepsis on days 1 and 5 of nutritional support. Specimens were collected from the bottom layers of fresh stools of the patients with a sampling spatula after spontaneous defecation or a warm saline enema. The stool specimens were quickly placed in stool specimen boxes, which were enveloped, labeled, and stored in liquid nitrogen tanks and transferred to a -80 °C refrigerator for freezing.

Each stool sample was spiked with 790 μL of lysis solution [4M guanidine thiocyanate, 250 μL; 10% N-lauroyl sarcosine, 40 μL; 5% N-lauroyl sarcosine-0.1 M phosphate buffer (pH 8.0), 500 μL], followed by a vigorous vortex mixing, and then incubated at 70 °C for 1 h. At the end of the incubation, bead-beating with glass beads (0.1 mm, 500–750 μL) was performed for 10 min (25HZ/S); subsequent extraction was performed according to the instructions of the extraction kit (E.Z.N.A.® Stool DNA Kit).

The concentration of extracted DNA was measured using NanoDrop (Thermo Scientific). The V3-V4 region of the 16S rRNA gene was amplified by primers 341F/805R (341F: *5'-CCTACGGGNGGCWGCAG-3'*; 805R: *5'-GACTACHVGGGTATCTAATCC-3'*), and the PCR products were sequenced using an Illumina Miseq 2 × 300 bp platform.

Short-chain fatty acid metabolism test

Standard configuration: Appropriate amounts of pure acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and caproic acid were used to prepare 10 mixed concentration gradients (0.02 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 2 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 250 μg/mL, and 500 μg/mL). The master mixes and working standard solutions were stored at 0 °C.

Sample pre-treatment: Appropriate amount of sample was added along with 50 μL of 15% phosphoric acid, 100 μL of 125 μg/mL of internal standard (isocaproic acid), and 400 μL of ether. The solution was then homogenized for 1 min, centrifuged at 4 °C for 10 min at 12000 rpm, and the supernatant was collected for testing.

GC-MS detection method: The chromatographic conditions were as follows: Agilent HP-INNOWAX capillary column (30 m × 0.25 mm ID × 0.25 μm); split injection, injection volume 1 μL, split ratio 10 : 1; inlet temperature 250 °C; ion source temperature (eng) 230 °C; transmission line temperature 250 °C, quadrupole temperature 150 °C. The temperature ramp-up time was programmed to start at 90 °C, ramping up to 120 °C at 10 °C/min, to 150 °C at 5 °C/min, and ultimately reaching 250 °C at 25 °C/min for 2 min. The carrier gas was helium at a flow rate of 1.0 mL/min.

Blood test methods

Lymphocyte subpopulation analysis: Flow cytometry method, contained T lymphocytes (CD3+), B lymphocytes (CD3- CD19+), and NK lymphocytes (natural killer cells, CD3- CD16+ and/or CD56+); T cells were subdivided into helper T cells (CD3+ CD4+) and suppressor T cells (CD3+ CD8+). The DxFLEX flow cytometry (lymphocyte division) analyzer (Beckman Coulter Ltd., United States) and lymphocyte assay kit (Beckman Coulter Ltd., United States) were used.

Immunoglobulin + complement, nutritional panel (scattering and turbidimetric method): The nutritional panel contained albumin (ALB), prealbumin (PAB), and transferrin; immunoglobulin and the complement tests contained immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), C3, and C4. BN II analyzer (SIEMENS GmbH, Germany). The IgM assay kit as well as the nutritional complete assay kit (SIEMENS Healthcare Diagnostics GmbH, Germany) were used.

Statistical methods

Clinical data were processed and analyzed using the Statistical Package for Social Sciences version 19.0 (IBM, Armonk, New York), and the data were first tested for normality and variance. Normally distributed variables and variance measures were expressed as mean ± SD, using *t*-test and analysis of variance; non-normally distributed measures were expressed as M (P25, P75), using a rank sum test; χ^2 test was used for the comparison of frequency data. A P value < 0.05 was considered statistically significant.

Raw sequencing data were processed using USEARCH (version 11.0.667) and operational taxonomic units (OTUs) were classified based on 97% sequence similarity, and representative OTU sequences were compared with the SILVA database (SSU138, http://www.arb-silva.de) to obtain the taxonomic classification of each 16S rDNA sequence. The α-diversity of each sample was assessed using Ace, Chao 1, Shannon-Wiener diversity index, and Simpson diversity index, and differences between the groups were tested using the non-parametric Mann–Whitney U test or Kruskal–Wallis rank sum test. Principal coordinates analysis and Linear discriminant analysis Effect Size analysis were performed and heat maps were constructed using R statistical software. Spearman correlation analysis was performed on gut microbiota, SCFA metabolism, and clinical nutritional and immunological indicators of the patients with sepsis treated with different modalities of nutritional support for 5 d.

RESULTS

Characteristics of the participants

Patients with sepsis were grouped into TEN, TPN, and SPN groups according to different nutritional modalities, and basic patient data were compared (Table 1). There were no significant differences between the groups in terms of sex, age, underlying disease, and body mass index (P > 0.05). The APACHE II and SOFA scores were compared among the three groups, and no significant differences were observed in the degree of severity of sepsis (P > 0.05). Furthermore, the NUTRIC score was ≥ 6 in all three groups, and no significant differences were observed (P > 0.05). The daily REE of patients with

Table 1 Comparison of the general information among the three groups of individuals

Indicator	TEN (*n* = 10)	TPN (*n* = 10)	SPN (*n* = 10)	H/F/χ^2/Z/*t* value	*P* value
Age (yr, mean ± SD)	59.20 ± 12.52	54.30 ± 15.10	59.30 ± 16.33	0.46	0.63
Sex					
Male (case, %)	8 (80)	8 (80)	8 (80)	0.00	1.00
Female (case, %)	2 (20)	2 (20)	2 (20)		
Underlying disease					
Hypertension (case, %)	4 (40)	3 (30)	5 (50)	0.83	0.65
Diabetes (case, %)	2 (20)	4 (40)	2 (20)	1.36	0.50
Malignant tumor (case, %)	0 (0)	2 (20)	2 (20)	2.30	0.31
APACHE II score on day 1 (points, mean ± SD)	14.10 ± 2.84	15.30 ± 4.73	19.50 ± 7.38	2.12	0.14
SOFA score on day 1 (points, mean ± SD)	5.40 ± 1.51	9.60 ± 1.95	10.20 ± 4.10	5.53	0.10
BMI (kg/m^2, mean ± SD)	23.90 ± 2.79	26.84 ± 3.89	26.05 ± 2.67	1.90	0.17
NUTRIC score on day 1					
≥ 6 points (case, %)	3 (30)	6 (60)	6 (60)	2.40	0.30
< 6 points (case, %)	7 (70)	4 (40)	4 (40)		
REE on day 1 [kcal, M (P25, P75)]	1950.00 (1759.28, 2050.72)	1900 (1726.40, 2073.59)	2036 (1882.46, 2189.53)	2.49	0.28

APACHE II score: Acute Physiologic Assessment and Chronic Health Evaluation II Scoring System; SOFA score: Sequential Organ Failure Assessment Score; BMI: Body mass index; NUTRIC score: Nutritional Risk Rating Instrument for Critically Ill Patients; REE: Resting energy expenditure; TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition.

sepsis obtained by indirect calorimetry performed by the ventilation circuit was used as the set amount for nutritional support, and no significant difference was observed among the three groups in the 24-h REE ($P > 0.05$). General information were balanced and comparable between the groups.

Effect of different nutritional modalities on gut microbiota in patients with sepsis

No significant improvement in α-diversity and β-diversity of gut microbiota was observed in the three groups, before and after nutritional support (Supplementary Figures 1 and 2) ($P > 0.05$).

Comparison of gut microbiota composition at the genus and OTU levels: The Mann-Whitney U test was separately performed in each group to compare the genera of the gut microbiota before and after nutritional support. Results indicated that compared with the gut microbiota of patients with sepsis before nutritional support, significant changes occurred in some genera of the gut microbiota after 5 d of nutritional support. Specifically, Enterococcus was significantly higher in the TEN group after nutritional support ($P < 0.05$) (Figure 1A); Campylobacter was significantly lower in the TPN group after nutritional support ($P < 0.05$) (Figure 1B); and Dialister was significantly lower in the SPN group after nutritional support ($P < 0.05$) (Figure 1C).

A random forest approach was used to compare the OTU level of the three groups, and changes in key bacterial OTUs before and after nutritional support were identified. After nutritional support in the TEN group, we obtained a significant reduction in the relative abundance of 10 OTUs, including those belonging to the genera Streptococcus, Methylobacterium-Methylorubrum, Oscillospiraceae *UCG-005*, Faecalibacterium, Escherichia-Shigella, Eubacterium coprostanoligenes group, Agathobacter, and Hungatella. Furthermore, the relative abundance of one OTU belonging to the genus Enterococcus and one unclassified OTU belonging to the Enterobacteriaceae family significantly increased (Figure 2A). After nutritional support in the TPN group, we found a significant reduction in the relative abundance of 16 OTUs, including those belonging to the genera Anaerococcus, Prevotella, Porphyromonas, Methylobacterium-Methylorubrum (2 OTUs), Faecalibacterium (2 OTUs), Ochrobactrum, Sphingomonas, Phyllobacterium, Agathobacter, Blautia, Pelomonas, and Acidovorax, while the relative abundance of the two OTUs belonging to the genus Enterococcus significantly increased (Figure 2B). After nutritional support in the SPN group, we found a significant reduction in the relative abundance of 11 OTUs, belonging to the genera Dialister, Serratia, Streptococcus, Porphyromonas, Anaerostipes, Veillonella, Fusicatenibacter, Escherichia-Shigella, and Ezakiella, and two unclassified OTUs in the families Lachnospiraceae and Ruminococcaceae (Figure 2C).

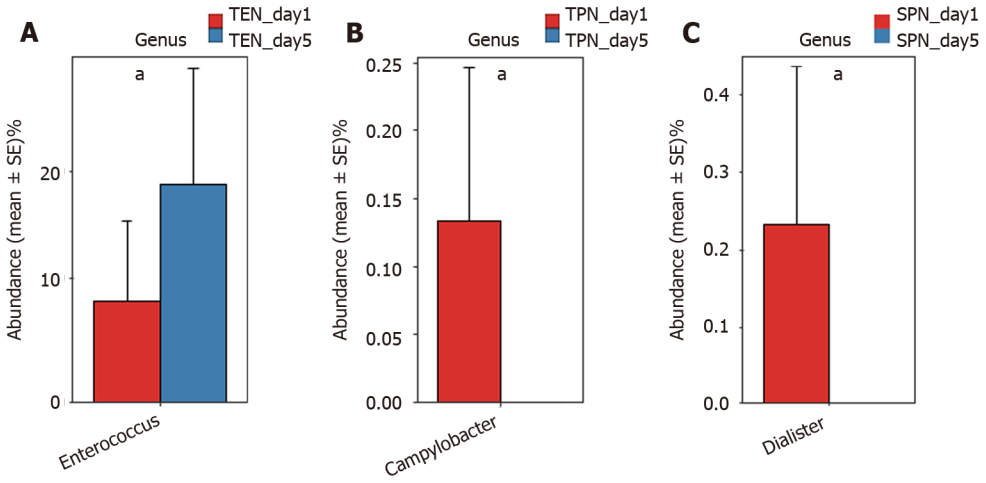

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Figure 1 The key bacterial taxa significantly changed before and after nutrition support at the genus level. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Total enteral nutrition; B: Total parenteral nutrition; C: Supplemental parenteral nutrition. The relative abundance of each genus is depicted as the mean ± SE. *P* values were calculated using the Mann–Whitney U test: [a]*P* < 0.05. TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition.

Effect of different nutritional modalities on short-chain fatty acid metabolism in patients with sepsis

The GC-MS assay was used to detect fecal SCFAs before and after nutritional support in the three groups. The changes in the SCFAs before and after nutrition in the TEN (Figure 3), TPN (Figure 4), and SPN (Figure 5) groups were compared and all SCFAs in the TEN group showed an increasing trend except for caproic acid. Acetic and propionic acid in the TPN group showed an increasing trend, while all SCFAs in the SPN group showed a decreasing trend. However, no significant difference ($P > 0.05$) was observed in the changes of SCFAs by nutritional support pathways in patients with sepsis, both before and after group nutrition and between groups.

Effect of different nutritional modalities on immune and nutritional indicators in patients with sepsis

Paired sample *t*-test and non-parametric test for correlated samples were used to compare the immune and nutritional indicators of the three groups before and after nutrition. The results of these analyses are shown in Table 2. After nutritional support in the TEN group, CD3+ T cells, CD3+/CD4+ T cells, IgG, complement C3, PAB, and ALB levels significantly increased, while CD3- CD19+ B cells significantly decreased ($P \leq 0.05$). In the TPN group, only the IgG level significantly increased after nutritional support ($P = 0.007$), while the remaining immune and nutritional indicators did not change significantly ($P > 0.05$). In the SPN group, IgA, IgG, complement C3, PAB, ALB, and transferrin levels significantly increased, while CD3- CD19+ B cell levels significantly decreased after nutritional support ($P \leq 0.05$).

To avoid the influence of immune and nutritional indicators of patients in each group before nutritional support on indicators after nutrition, analysis of covariance was used to compare the three groups. Post-nutritional pre-ALB was significantly higher in the TEN group than in the TPN group ($P = 0.05$), while ALB was significantly higher in the SPN group than in the TPN group ($P = 0.019$).

Correlation analysis between gut bacteria abundance and SCFAs metabolism as well as nutritional immune indicators after nutritional support for patients with sepsis

Spearman correlation analysis revealed that after nutritional support there was a correlation between the more dominant species in the gut and SCFAs and clinical immune and nutritional indicators in patients with sepsis (Figure 6). For example, Enterococcus spp., which increased in abundance in the TEN group after the nutritional intervention, showed a significant positive correlation with IgA; Streptococcus and Escherichia-Shigella spp., which decreased in abundance, showed a significant negative correlation with PAB concentrations, as well as a significant positive correlation with intestinal concentrations of multiple SCFAs. Methylobacterium spp. were significantly negatively correlated with IgG and SCFA concentrations (all $P < 0.05$). Anaerococcus and Porphyromonas spp., which decreased in the TPN group, showed a significant positive correlation with PAB concentrations, and Methylobacterium, Sphingomonas, and Phyllobacterium spp. showed a significant negative correlation with IgG and multiple SCFAs concentrations (all $P < 0.05$). Streptococcus and Escherichia-Shigella, which showed decreased abundance in the SPN group, were negatively correlated with PAB concentrations and also positively correlated with multiple SCFA concentrations; Porphyromonas and Anaerostipes were positively correlated with PAB concentrations (all $P < 0.05$). In addition, no significant correlations were observed between clinical nutritional immune indicators such as ALB, CD3+ T cells, CD3- CD19+ B

Table 2 Effect of different nutritional modalities on immune and nutritional indicators in patients with sepsis

Indicator	TEN (*n* = 20)				SPN (*n* = 20)				TPN (*n* = 20)			
	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value	Day 1 (*n* = 10)	Day 5 (*n* = 10)	*t*/Z value	*P* value
CD3+ T cell [%, M (P25, P75)]	69.85 (63.62, 78.77)	77.77 (69.40, 80.48)	2.09	0.03	71.33 (64.42, 76.50)	72.25 (64.59, 82.59)	1.78	0.07	74.21 (59.82, 83.43)	74.80 (67.23, 82.74)	0.76	0.44
CD3+ CD4+ T cell [%, M (P25, P75)]	36.40 (29.47, 43.39)	40.39 (36.96, 46.50)	1.98	0.04	38.21 (32.22, 57.15)	47.37 (35.61, 58.27)	1.17	0.24	38.12 (29.23, 46.40)	45.67 (28.13, 51.69)	1.37	0.16
CD19+ B cell [%, M (P25, P75)]	17.04 (11.55, 21.27)	14.11 (8.99, 18.03)	1.98	0.04	16.80 (10.70, 25.14)	9.75 (6.47, 16.59)	2.09	0.03	14.35 (6.62, 22.89)	12.78 (6.93, 23.61)	1.02	0.30
IgA (g/L mean ± SD)	2.53 ± 1.18	2.93 ± 0.93	1.97	0.08	2.15 ± 0.83	2.91 ± 1.19	3.87	0.00	2.10 ± 1.13	2.45 ± 0.73	1.44	0.18
IgG (g/L, mean ± SD)	11.75 ± 3.46	15.19 ± 5.84	3.10	0.01	9.05 ± 3.78	13.77 ± 4.52	5.48	0.00	9.32 ± 3.89	12.15 ± 3.85	3.48	0.00
IgM (g/L, mean ± SD)	0.81 ± 0.36	1.11 ± 0.48	1.49	0.16	0.95 ± 0.44	1.12 ± 0.75	1.11	0.29	0.77 ± 3.89	0.89 ± 0.46	1.02	0.33
C3 (g/L, mean ± SD)	0.99 ± 0.30	1.21 ± 0.23	2.84	0.01	0.81 ± 0.21	1.07 ± 0.23	4.73	0.00	0.77 ± 0.25	0.88 ± 0.33	0.95	0.36
C4 (g/L, mean ± SD)	0.19 ± 0.04	0.22 ± 0.05	2.08	0.06	0.19 ± 0.07	0.22 ± 0.07	3.64	0.00	0.17 ± 0.05	0.21 ± 0.08	1.75	0.11
Albumin (g/L, mean ± SD)	27.14 ± 3.18	29.89 ± 3.61	3.04	0.01	27.74 ± 3.45	30.97 ± 2.63[a]	2.68	0.02	28.10 ± 3.37	27.73 ± 2.64	0.24	0.81
Pre-albumin (g/L, mean ± SD)	0.10 ± 0.04	0.15 ± 0.07[a]	3.88	0.00	0.08 ± 0.41	0.13 ± 0.05	3.42	0.00	0.10 ± 0.03	0.11 ± 0.03	0.47	0.64
Transferrin (g/L, mean ± SD)	0.01 ± 0.28	0.01 ± 0.28	1.52	0.15	1.16 ± 0.36	1.36 ± 0.51	2.67	0.02	1.05 ± 0.30	1.20 ± 0.37	1.11	0.29

[a]$P \leq 0.05$ compared with the enteral nutrition group.

TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition; day 1: Before nutrition support; day 5: After nutrition support; IgA: Immunoglobulin A; IgM: Immunoglobulin M; IgG: Immunoglobulin G.

cells, gut bacteria, and SCFAs (all $P > 0.05$).

DISCUSSION

Effect of different nutritional modalities on gut microbiota diversity and species composition in patients with sepsis

By comparing the gut microbiota of septic patients, non-septic patients, and healthy controls, our previous study confirmed that septic patients undergo gut microbiota disorders, which persist for a week or longer[3]. This is consistent with the current knowledge of the characteristics of gut microbiota disorders in patients with sepsis[7,8]. Gut microbiota disorders contribute to the development of sepsis through the proliferation of pathogenic bacteria, dysregulated immune response, and reduced production of microbiota-derived metabolites, such as SCFAs. After the onset of sepsis, alterations in the structure of the normal gut microbiota can exacerbate the condition, leading to a worsening of the prognosis for sepsis, and the development of MODS[4].

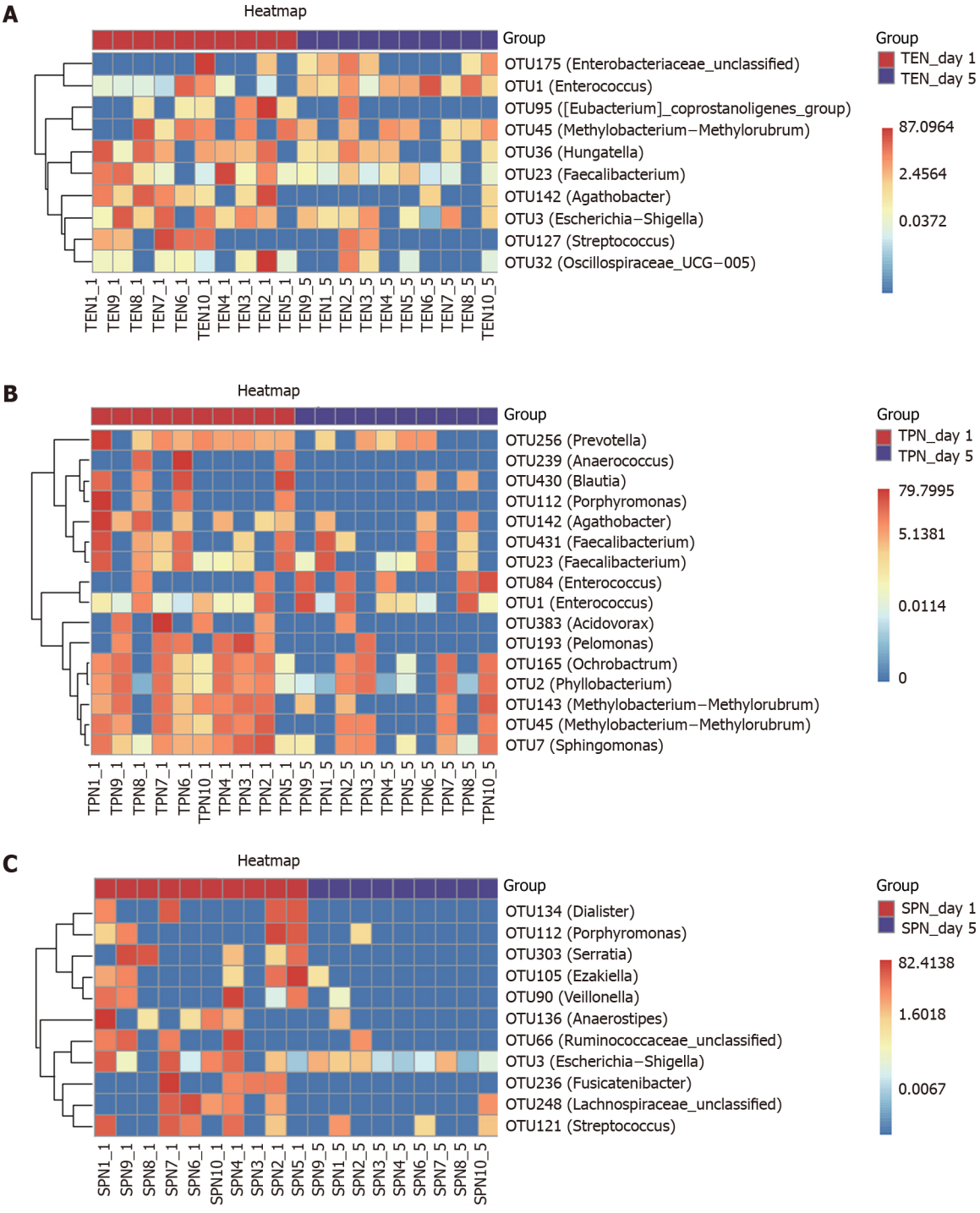

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Figure 2 Heatmaps display the key gut microbial operational taxonomic units influenced by three types of nutritional support. A: Total enteral nutrition; B: Total parenteral nutrition; C: Supplemental parenteral nutrition. The key operational taxonomic units are selected by Random Forest approach according to the changes of relative abundance before and after nutritional support. TEN: Total enteral nutrition; TPN: Total parenteral nutrition; SPN: Supplemental parenteral nutrition; OTU: Operational taxonomic unit.

Nutritional support is an important part of the treatment of critically ill patients. Diet is by far the most significant among the factors known to affect the gut microbiota[9,10]. Studies have found that diet under normal physiological conditions can affect the gut flora in the short-term, rapidly, and significantly[11]. In this study, we found that nutritional support for 5 d using different modalities of nutritional support did not show significant improvement in the gut microbiota disorders of patients with sepsis. This is in line with the perception of gut microbiota disorders in sepsis. Although the mechanism of intestinal microecological disorders due to sepsis is not clear, multiple factors such as antibiotics[12], sedative and analgesic drugs[13], nutritional support[14], and proton pump inhibitors [15] affect gut flora among patients with sepsis, making it impossible for a single factor to significantly

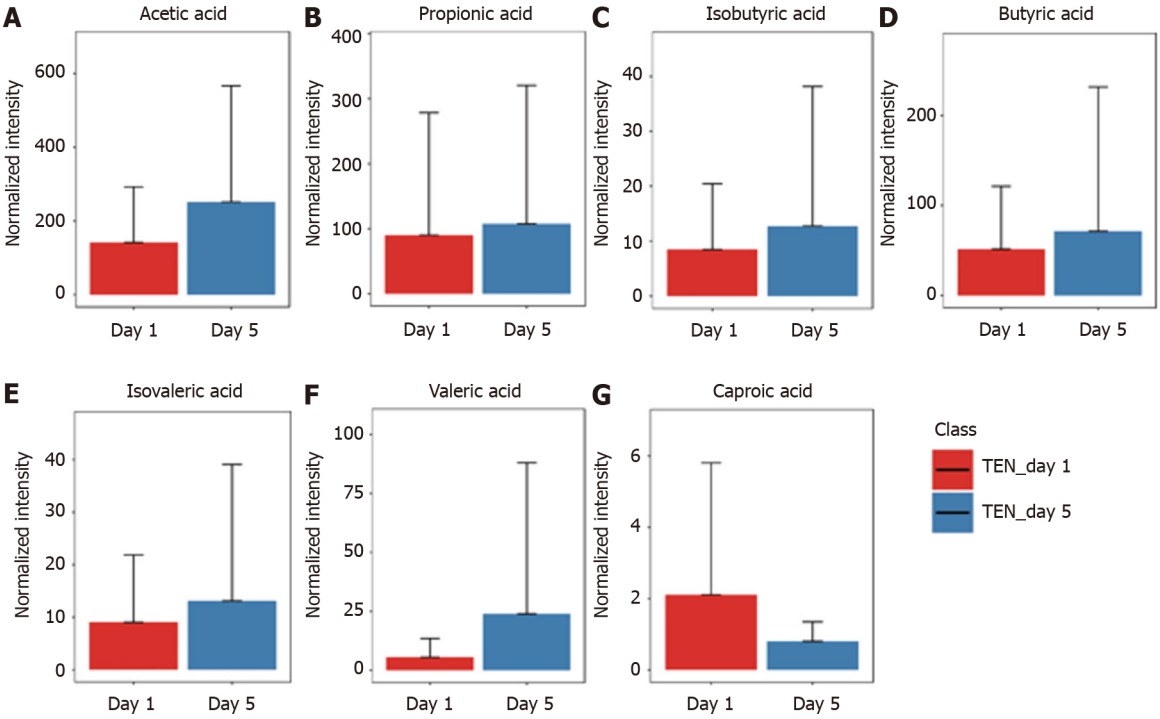

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Figure 3 The changes of short-chain fatty acids before and after nutrition support in the total enteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. TEN: Total enteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD.

improve the condition in a short period of time.

Although short-term nutritional support could not correct gut microbiota disorders in patients with sepsis, different nutritional support modalities caused some differences in bacterial species: Enterococcus spp. were significantly high in the TEN group, Campylobacter spp. were significantly low in the TPN group, and Dialister spp. were significantly low in the SPN group. In animal studies, it has been demonstrated that Enterococcus can effectively improve animal growth[16,17], enhance the absorption of other nutrients[18], and is a beneficial additive, which is widely used in animal feeds. Current research suggests that drug-resistant strains of Enterococcus may be pathogenic[19]; however, its commensal strains have been identified as probiotics in animal and human intestines[20]. Further studies have shown that Enterococcus can trigger immune signaling pathways and regulate infection [21], autoimmunity[22]. Therefore, we believe that a significant increase of Enterococcus spp. after TEN treatment suggests that early TEN support can improve gut microbiota in sepsis. Campylobacter spp. is the main cause of gastroenteritis in humans, and consumption of contaminated poultry meat is its main transmission route. The significant decrease of this genus after TPN treatment suggests improvements in gut microbiota in sepsis; however, the underlying mechanism is currently unclear and requires further study. Non-oral ingestion during TPN support may have cut-off the transmission route of Campylobacter spp. Other reports suggest that a variety of probiotic bacteria in the intestine reduce the severity of Campylobacter spp. infection by negatively affecting the virulence and survival factors (*e.g.*, adhesion, invasion) of various Campylobacter spp., attenuating intestinal inflammation[23]. Dialister spp. may be positively associated with glucose metabolism disorders, obesity, and insulin resistance[24, 25], and our previous study found that Dialister spp. in patients with sepsis was positively associated with diamine oxidase, a highly active intracellular enzyme in the upper villi of the small intestinal mucosa in humans and mammals. Elevated diamine oxidase reflects the degree of damage to the intestinal mechanical barrier[4]. Therefore, the significant decrease in Dialisteria spp. suggests that SPN may improve intestinal barrier function.

Early, short-term nutritional support in sepsis has a beneficial effect on gut bacteria composition, although it did not completely improve gut microbiota disorders.

Effects of different nutritional modalities on SCFAs in patients with sepsis

SCFAs are the main metabolites produced by specific symbiotic bacteria of the gut after fermentation of dietary fiber and indigestible polysaccharides and starches, including acetic, propionic, butyric, isobutyric, pentanoic, isovaleric, and caproic acid. Among them, acetic, propionic, butyric, and isovaleric acid are the most common SCFAs. In addition to their ability to provide direct nutrition to intestinal commensal microorganisms, SCFAs positively influence host immune cell differentiation,

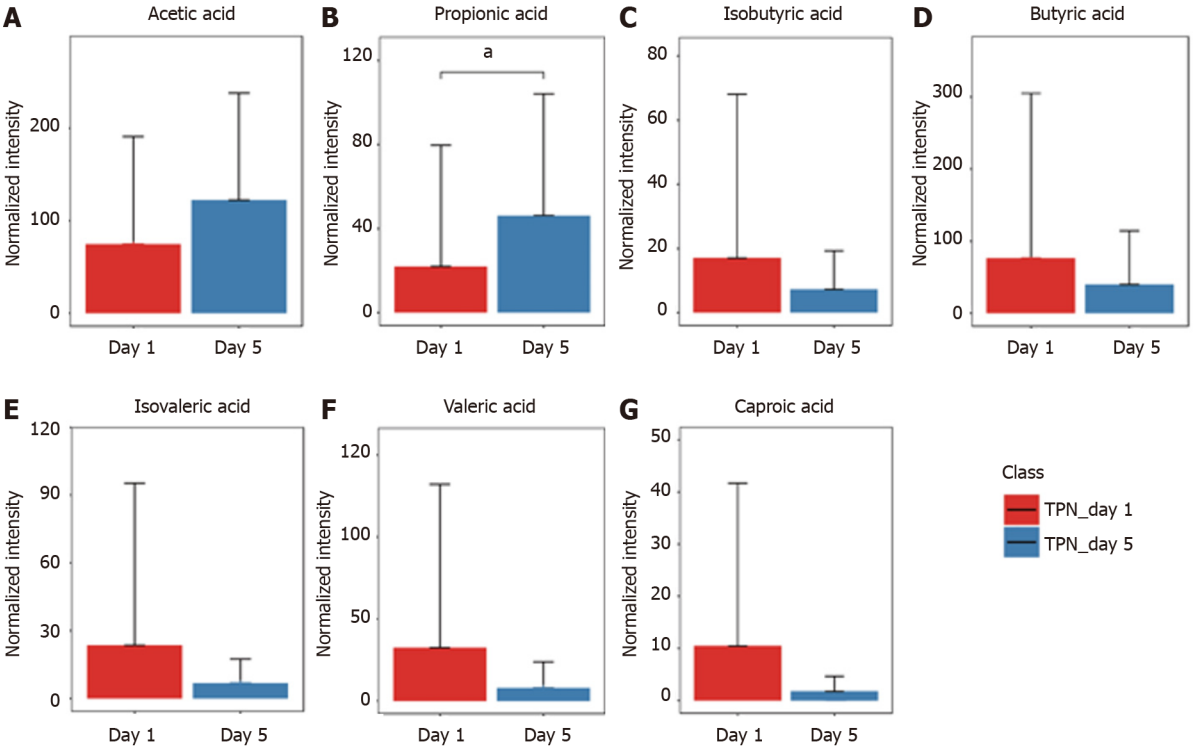

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Figure 4 The changes of short-chain fatty acids before and after nutrition support in the total parenteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. TPN: Total parenteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD. *P* values were calculated using the Student's *t*-test: $^{a}P < 0.05$.

immune system, and metabolism as well as modulate host susceptibility[26,27]. Furthermore, they have a protective effect on intestinal barrier function[28,29].

In this study, we found that there was no significant improvement in SCFAs after 5 d of nutritional support in patients with sepsis. This is complemented by the fact that gut microbiota disorders in sepsis are not effectively corrected, and a close correlation exists between SCFAs and gut microbiota, and SCFAs cannot be significantly improved in the absence of effective correction of gut microbiota disorders. In patients with sepsis, there is a significant decrease in SCFA-producing bacteria in the intestine, such as Bifidobacterium and Bacillus. This can cause a dramatic decrease in SCFAs and cause intestinal barrier dysfunction. Concomitantly, decreased levels of SCFAs can further aggravate the gut microbiota disorders in sepsis[30,31].

In this study, we found that all SCFAs in the TEN group showed an increase trend except for caproic acid. In the TPN group, acetic acid showed an improving trend and propionic acid improved significantly, while the remaining SCFAs did not show any improvement. SCFAs in the SPN group showed a continuous decreasing trend. The complete bypassing of the intestine by TPN can serve as both advantage and disadvantage[32]. TPN lacks key intestinal luminal nutrients compared to TEN, resulting in a significant deficiency of glutamine and SCFAs[33,34]. Animal studies have shown that this deficiency cannot be effectively improved by the addition of glutamine and SCFAs to intravenous nutrition[34-36]. Supplemental enteral nutrition during SPN mostly uses ready-made enteral preparations with low fiber or without fiber, which not only fails to promote the production of SCFAs but can also form pathogenic microorganisms with higher virulence[30,37]. The reason for this may be the inability of highly absorbable trophic preparations to adequately feed the intestinal epithelium and intestinal commensal bacteria, leading to disruption of the intestinal barrier, stress signaling, and proliferation of intestinal pathogenic bacteria[38].

Although nutritional support in early sepsis did not significantly improve SCFA metabolism, different nutritional support modalities had different effects on SCFAs. Moreover, TEN had a tendency to improve overall SCFA production in sepsis compared to TPN and SPN and was theoretically more conducive to SCFA production due to dietary fiber supplementation and adequate enteral nutritional supplementation.

Effects of different nutritional modalities on nutritional and immunological indicators in patients with sepsis

In this study, we found that in early and short-term nutritional support for patients with sepsis, TEN

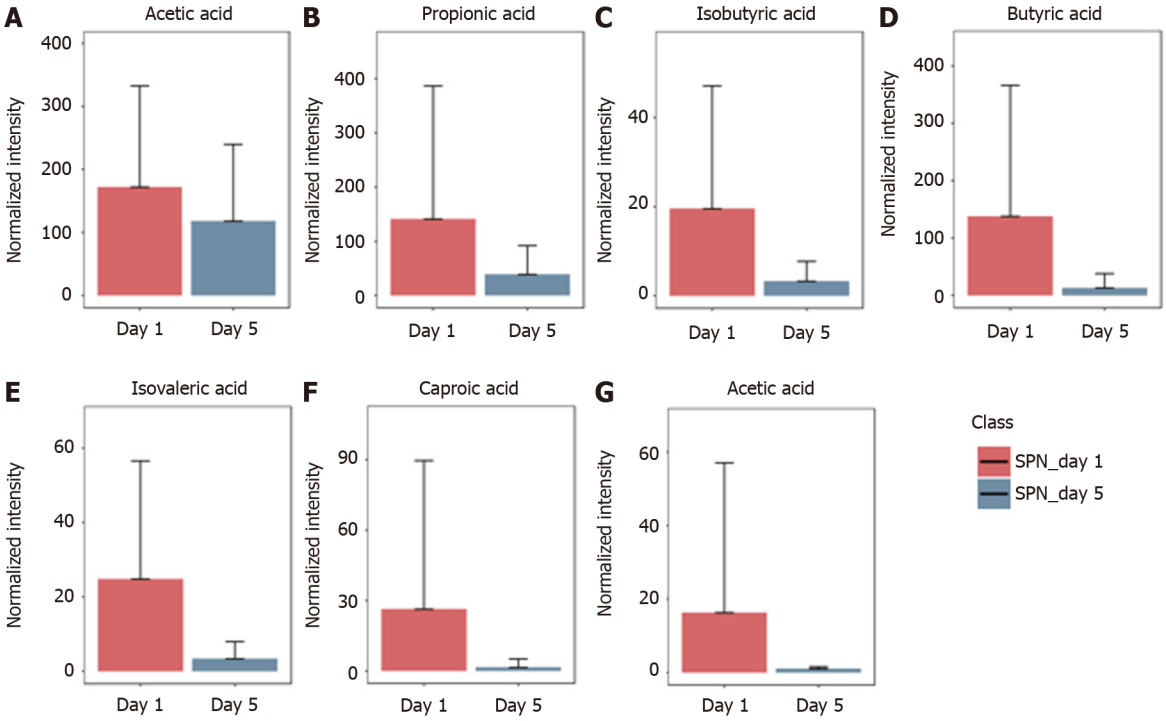

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Figure 5 The changes of short-chain fatty acids before and after nutrition support in the supplemental parenteral nutrition group. [Red: Before nutrition support (day 1); blue: After nutrition support (day 5)]. A: Acetic acid; B: Propionic acid; C: Isobutyric acid; D: Butyric acid; E: Isovaleric acid; F: Valeric acid; G: Caproic acid. SPN: Supplemental parenteral nutrition. The abundance of each short-chain fatty acid is depicted as the mean ± SD.

can significantly improve cellular immunity, humoral immunity, and nutrition-related indicators; SPN can significantly improve humoral immunity and nutritional indicators; and TPN can only improve IgG. The effects of the disease, vascular leakage, fluid supplementation, diuresis, bed confinement, and other factors, make weight assessment difficult for patients with sepsis. Additionally, muscle mass and other assessment indicators do not truly reflect the nutritional changes of the patient. Therefore, it is more appropriate to select plasma proteins as an indicator of nutrition in patients with sepsis. These proteins also need to have relatively small storage, rapid synthesis, and stable metabolic rate. Pre-ALB has a half-life of only 48 h, a small body storage pool, responds to negative nitrogen balance in the body in a short time, and is a considerably more sensitive indicator of malnutrition than either ALB or transferrin. Combined with the effect on PAB in the three groups in this study, early nutritional support of TEN in sepsis significantly improved the nutritional status of patients compared with TPN. Interestingly, PAB concentration was negatively correlated with the relative abundance of OTUs of Streptococcus and Escherichia-Shigella, while it was positively correlated with the relative abundance of OTUs of Anaerococcus and Porphyromonas. Additionally, the corresponding species were significantly lower in TEN and TPN, respectively. Further studies are needed to clarify whether this may suggest a relationship between improved nutrition and gut microbiota.

The intestine is the largest immune organ in the body. Studies in mice have shown that nutritional pathways and typology can affect gut-associated lymphoid tissue[39]. Relative to TPN, TEN reduces the occurrence of potential gastrointestinal complications, including maintenance of the intestinal epithelial barrier and tight junctions, increases constituent proteins, reduces acute phase reactants and bacterial translocation, and enhances mucosal immunity[40]. A meta-analysis showed that enteral feeding and sepsis are negatively correlated[41]. A more plausible explanation at present is that TPN lacks intraluminal nutrients, such as glutamine and SCFAs, which are essential for local enterocytes and colon cells, and that delivery of these nutrients *via* extra-gastrointestinal routes does not directly deliver nutrients to the most nutrient-demanding cells of the gastrointestinal tract. In contrast, key intestinal-systemic signaling regulators were significantly downregulated in animals fed *via* TPN[34]. These findings explain why TEN and SPN in this study could improve nutritional and immune indicators early in patients with sepsis, while TPN did not significantly improve these indicators. Related studies have also shown that, in addition to the nutritional pathway, the composition and adequacy of nutrients in the intestinal lumen determine the gene expression of host intestinal receptors as well as the uptake of luminal metabolites and hormonal signaling[36]. Approximately 20% of the energy supply of SPN passes through the gastrointestinal tract, which allows early improvement of nutrition and humoral immunity in patients with sepsis; however, due to the single formulation of the provided preparation, the frequent absence of dietary fiber, and the insufficient total energy supply through the intestine, SPN

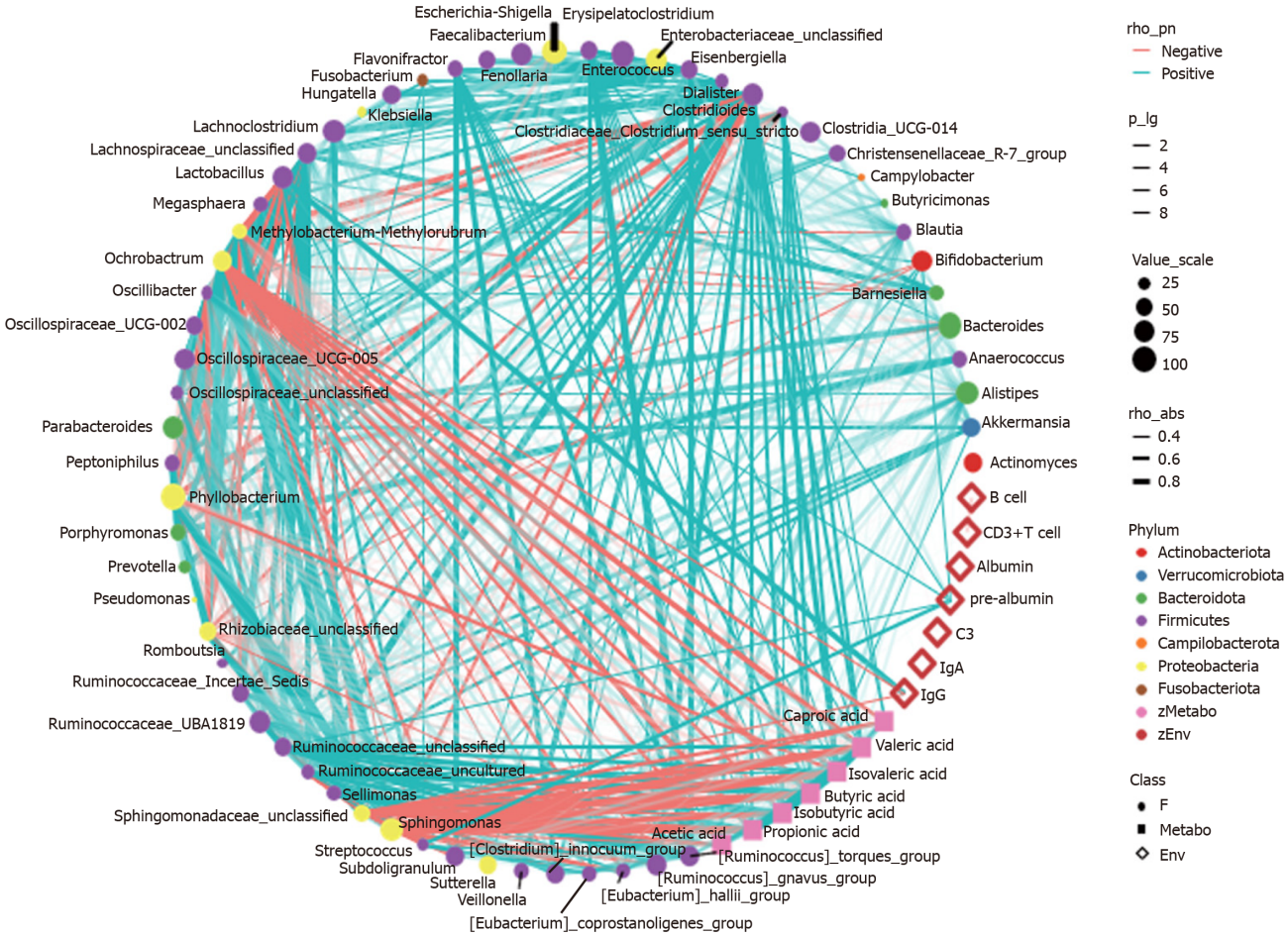

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Figure 6 Correlation analysis between gut microbiomes, short-chain fatty acids and nutritional immune indicators after nutritional support for patients with sepsis. Network graph constructs based on Spearman's correlation analysis. The results showed correlations among 60 predominant fecal microbial genera, 7 short-chain fatty acids (SCFAs) and 7 clinical immune and nutritional indicators. Connecting lines represent the significant correlation ($P < 0.05$). Red lines indicate negative correlations, blue lines indicate positive correlations, and the width of the lines represents the strength of the correlation. The transparency of the lines represented the negative logarithm of the *P* value of correlation. The size of the points indicates the relative abundance of genera. The colors of points display the different phyla of the microbiome. The circle represents the fecal microbiome, the square represents the SCFAs, and the diamond represents plasma metabolites.

does not improve cellular immunity and SCFAs during early nutritional support in sepsis.

IgG and IgA are important immunoglobulins that interact with the gut microbiota to exert corresponding immune effects[42]. In this study, the intestinal Enterococcus spp. and IgA were positively correlated and Methylobacterium spp. and IgG were negatively correlated in patients with sepsis. Accordingly, these two species also showed significant increase and decrease in the TEN group, which suggested interaction between IgG, IgA, and gut microbiota. Meanwhile, the correlation between Enterococcus spp. and IgA may be one of the mechanisms by which Enterococcus spp. exert beneficial effects on the intestinal microecology in sepsis, which remains to be further investigated.

The present study is a preliminary study investigating the modalities of early nutritional support in patients with sepsis from the perspective of intestinal microecology. However, this study is limited by the small sample size and short duration of nutritional support; therefore, significant improvements in intestinal microecology did not have time to materialize. The only differences are observed in the bacterial genera and changes in SCFAs. However, the available preliminary results provide an insight into the effects of different modalities of nutritional support on intestinal microecology, and their link to host nutrition and immunity, suggesting that TEN may be a more appropriate approach for early nutritional support in patients with sepsis. How different nutritional support modalities affect the structural and metabolic changes of gut microbiota composition and the related mechanisms involved need to be studied and explored by designing clinical trials with larger scale and longer intervention time, which is the next direction of our research.

CONCLUSION

In this study, three groups were classified on the basis of different modalities of nutritional support for sepsis. By comparing the changes in gut microbiota structure, species composition, and SCFAs, early, short-term nutritional support could not completely improve intestinal microecological disorders in sepsis, and accordingly, there was no significant improvement in SCFAs. However, the differences in the species composition of intestinal bacteria suggested a beneficial effect of early nutritional support on gut microbiota disorders. Relative changes in SCFAs showed that TEN support had an improving trend on the indicators. The changes in clinical nutritional and immune indicators before and after nutritional support showed that early TEN support largely improved cellular immunity, humoral immunity, and nutritional indicators. Moreover, TEN support improved clinical nutritional indicators of patients better than TPN. Meanwhile, the correlation between gut microbiota and SCFAs and clinical nutritional and immune indicators suggested some level of interaction. In conclusion, the results of this study suggest that TEN should be the recommended modality for early nutritional support in sepsis from the perspective of intestinal microecology. These findings offer a novel perspective for optimizing the mode of nutritional support in sepsis.

ARTICLE HIGHLIGHTS

Research background

Sepsis is a common disease in intensive care units, with high morbidity and mortality. Our previous study confirmed the presence of gut microbiota disorders in patients with sepsis. The combination of disease and gut microbiota disorders lead to the development of multiple organ dysfunction and clinical deterioration of the patient. Nutritional support is an important part of the treatment of critically ill patients. Proper modalities of nutritional support can improve nutrition, immunity, and intestinal microecology. The exploration of early nutritional support in patients with sepsis from the perspective of intestinal microecology is important to optimize nutritional support and improve prognosis.

Research motivation

The recommendations about the choice of nutritional support modalities are largely based on expert consensus, although the level of evidence is low. Nutritional support has a significant impact on intestinal microecology; to date, the modalities of nutritional support in sepsis have not been evaluated from the perspective of intestinal microecology. This perspective can provide new insights into the optimization of the modalities for nutritional support in sepsis.

Research objectives

The main objective was to determine the optimal modality of early nutritional support for patients with sepsis from the perspective of intestinal microecology. We applied different nutritional modalities for early and short-duration nutritional support in patients with sepsis and found differences in intestinal bacterial composition, short-chain fatty acids (SCFAs), and nutritional and immune indicators. Our results revealed for the first time that total enteral nutrition (TEN) is a good modality for early nutritional support in patients with sepsis. This study offers a new perspective for optimizing nutritional support modalities in sepsis.

Research methods

Thirty patients with sepsis who were admitted to the intensive care unit of the General Hospital of Ningxia Medical University, China, between 2019 and 2021 with indications for nutritional support, were randomly assigned to one of three different modalities of nutritional support. For 5 d, nutritional support was administered to each patient using one of the following modalities: TEN group, total parenteral nutrition (TPN group), and supplemental parenteral nutrition (SPN group). Blood and stool specimens were collected before and after nutritional support was administered, and changes in gut microbiota, SCFAs, and immune and nutritional indicators were detected and compared among the three groups.

Research results

Patients were assessed before and after the administration of nutritional support. The following differences were observed in the three groups after nutritional support: (1) Differences in the gut bacteria (Enterococcus increased in the TEN group, Campylobacter decreased in the TPN group, and Dialister decreased in the SPN group); (2) different trends in SCFA concentrations (increase in SCFAs in the TEN group except for caproic acid, improvement of acetic and propionic acid only in the TPN group, and decreasing trend in SCFA production in the SPN group); and (3) significant improvement in the nutritional and immunological indicators in the TEN and SPN groups, with improvement in immunoglobulin G levels only in the TPN group. Furthermore, and significant correlations were found

between the gut bacteria, SCFAs, and nutritional and immunological indicators.

Research conclusions

Our results indicate that TEN is the optimal modality for early nutritional support in patients with sepsis from the perspective of intestinal microecology.

Research perspectives

Future research should focus on how different nutritional support modalities affect the structural and metabolic changes in gut microbiota composition and the underlying mechanisms. Our research group will explore these questions in a large scale clinical trial with a longer intervention time.

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FOOTNOTES

Author contributions: Yang XJ was the guarantor and designed the study; Yang XJ participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Yang MY, Zhang XY, Liu QF, Yang G participated in the acquisition and the analysis of data; Wang XH, Ren HY, Chen H, Yang Y, revised the article critically for important intellectual content.

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Institutional review board statement: This study was approved by the Ethics Committee of General Hospital of Ningxia Medical University, No. 2017-266.

Clinical trial registration statement: This study is registered at http://www.chictr.org.cn. The registration identification number is ChiCTR2200066641.

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

Conflict-of-interest statement: Yang YJ reports grants from Ningxia Natural Science Foundation Project, grants from National Key Research and Development Program of China, during the conduct of the study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the SRA database of NCBI, SRA number: PRJNA861102. Participants consent for data sharing was not obtained but the presented data are anonymized and risk of identification is low. No additional data are available.

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SYSTEMATIC REVIEWS

Occam's razor or Hickam's dictum-COVID-19 is not a textbook aetiology of acute pancreatitis: A modified Naranjo Score appraisal

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Abstract

BACKGROUND

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe disease. During the coronavirus disease 2019 (COVID-19) pandemic, numerous reports of AP have been published, with most authors concluding a causal relationship between COVID-19 and AP. Retrospective case reports or small case series are unable to accurately determine the cause-effect relationship between COVID-19 and AP.

AIM

To establish whether COVID-19 is a cause of AP using the modified Naranjo scoring system.

METHODS

A systematic review was conducted on PubMed, World of Science and Embase for articles reporting COVID-19 and AP from inception to August 2021. Exclusion criteria were cases of AP which were not reported to be due to COVID-19 infection, age < 18 years old, review articles and retrospective cohort studies. The original 10-item Naranjo scoring system (total score 13) was devised to approximate the likelihood of a clinical presentation to be secondary to an adverse drug reaction. We modified the original scoring system into a 8-item modified Naranjo scoring system (total score 9) to determine the cause-effect relationship between COVID-19 and AP. A cumulative score was decided for each case presented in the included articles. Interpretation of the modified Naranjo scoring system is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable cause.

RESULTS

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67 articles were included in the final analysis, with 76 patients which had AP reported to be due to COVID-19. The mean age was 47.8 (range 18-94) years. Majority of patients (73.3%) had ≤ 7 d between onset of COVID-19 infection and diagnosis of AP. There were only 45 (59.2%) patients who had adequate investigations to rule out common aetiologies (gallstones, choledocholithiasis, alcohol, hypertriglyceridemia, hypercalcemia and trauma) of AP. Immunoglobulin G4 testing was conducted in 9 (13.5%) patients to rule out autoimmune AP. Only 5 (6.6%) patients underwent endoscopic ultrasound and/or magnetic resonance cholangiopancreatogram to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum. None of the patients had other recently diagnosed viral infections apart from COVID-19 infection, or underwent genetic testing to rule out hereditary AP. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP.

CONCLUSION

Current evidence is weak to establish a strong link between COVID-19 and AP. Investigations should be performed to rule out other causes of AP before establishing COVID-19 as an aetiology.

Key Words: COVID-19; Infections; Pancreatic diseases; Pancreatitis; Post-acute COVID-19 syndrome

Core Tip: Numerous reports of acute pancreatitis (AP) have been published during the coronavirus disease 2019 (COVID-19) pandemic, citing COVID-19 as an aetiology of AP. However, COVID-19 has not been well-established to be a cause of AP. A total of 76 patients were included in our systematic review and were assessed using the modified Naranjo score; there were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP. The link between COVID-19 and AP is weak based on current literature; COVID-19 should still remain a diagnosis of exclusion for AP until further evidence.

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INTRODUCTION

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe, with an incidence of 50-80 per 100000 population[1]. Gallstone disorders and alcohol abuse remain the two commonest global causes of AP[2]. In rare circumstances, AP may be triggered by viral or parasitic infections[3,4]. Recently, there have been reports of AP caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of the time of this article, the coronavirus disease 2019 (COVID-19) pandemic is responsible for over 642 million infections and 6.6 million deaths worldwide[5]. While COVID-19 is primarily a respiratory disease, patients with COVID-19 infection may experience extra-pulmonary symptoms[6]. There has been an increase in reports of autoimmune and inflammatory conditions attributed to SARS-CoV-2[7], one example of which is AP. While there have been sporadic case reports and attempts at literature reviews on the potential cases of COVID-19-induced AP, the significant increase in cases reported raises concerns regarding COVID-19 as a definitive causal etiology for AP rather than an epiphenomenon[8].

The distinction between "association" *vs* "causation" can only be derived by prospective longitudinal studies involving a large population or by rigorous statistical analysis of large datasets and registries. It remains unproven if published reports of AP (effect) are beyond doubt due to COVID-19 infection (causal etiology). This is similar to determining whether symptoms experienced following ingestion of a new medication may be coined as an "adverse drug reaction" and attributed to that drug. Naranjo *et al* [9] designed a questionnaire to determine the likelihood of an adverse drug reaction (effect) due to a drug (causal etiology) rather than associated confounding factors[9]. Recently, we have reported the utility of modified Naranjo score to diagnose AP in patients prescribed with sulphasalazine[10]. This is a novel study which aims to systematically review case series and/or reports on COVID-19-induced AP

and assess them using the modified Naranjo score to determine whether there is a cause-effect relationship.

MATERIALS AND METHODS

Study selection and search strategy

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines[11]. A literature search was performed on PubMed, World of Science and Embase for articles on COVID-19 and AP from inception till August 8, 2021. The following search terms were used: ((“COVID-19” OR “Coronavirus 19” OR “SARS-CoV-2”) AND (“Pancreatitis” OR “Acute Pancreatitis” OR “Acute Edematous Pancreatitis” OR “Pancreatic Inflammation”)); MeSH terms were used where available. Inclusion criteria were case series and/or case reports on AP, where the aetiology was thought to be COVID-19 infection. Exclusion criteria were (1) reports on AP but aetiology was not attributed to COVID-19 infection; (2) cases with patients < 18 years old; and (3) based on article type (non-English language, review articles and retrospective cohort and/or case-control studies). After removing of duplicates, three authors (Tang ZJT, Chua BQY, and Lim PK) independently screened the articles for potential inclusion by title and abstract. Subsequently, the full texts of screened studies were obtained and reviewed for eligibility. As some case reports may not have abstracts published, full texts were reviewed if abstracts were not available. Conflicts were resolved by appeal to the senior author.

The modified Naranjo score

The original Naranjo score was described by Naranjo *et al*[9] in 1981 as a means of approximating the likelihood of attributing a patient’s presentation to a medication and labelling it as an adverse drug reaction[9]. It consists of ten closed-ended questions with the options for “Yes”, “No,” and “Do not know”, each with varying points allocated from “- 1” to “+ 2”. The total score is 13 and may be interpreted as such: ≤ 0: Doubtful, 1-4: Possible; 5-8: Probably and ≥ 9: Definite. Existing studies have modified the Naranjo score to determine the cause-effect relationship in various pathologies[10]. In this review, we propose a modification to the Naranjo score to determine the likelihood of COVID-19-induced AP (Table 1). This is a 8-item scoring system with a total of 9 points; a cumulative score was calculated for each case, and a probability classification was assigned based on their score: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable.

Our modified version of the Naranjo score retained some of the questions used in the original study: Whether reports suggest a causal relationship, if the resolution of COVID-19 led to the resolution of AP, and ruling out alternative causes of AP. Common causes of AP were defined as gallstones, choledocholithiasis, alcohol, hypertriglyceridaemia, hypercalcaemia or trauma. Investigations for these included history and physical examination (for alcohol and trauma), biochemical investigations (for hypertriglyceridaemia and hypercalcemia), imaging studies (ultrasonography and/or computed tomography of the abdomen and pelvis) (for gallstones or choledocholiathisis) and endoscopic retrograde cholangiopancreatogram for choledocholithiasis in the presence of abnormal liver function test and/or biliary dilation. The original Naranjo score included the need for drug challenge test or placebo, but this was not appropriate for this study, as a “drug challenge” would imply re-introduction of COVID-19 infection and identifying if the patient had recurrence of AP. Instead, whether the resolution of infection led to the resolution of acute pancreatitis was included as a criterion.

We additionally included questions on whether there is a cause-effect temporal relationship and a more in-depth approach in ruling out the other aetiologies of AP. This included ruling out autoimmune pancreatitis with testing for serum Immunoglobulin G4 (IgG4)[12], or whether an endoscopic ultrasound (EUS) or magnetic resonance cholangiopancreatography (MRCP) was performed to rule out occult microlithiasis, pancreatic malignancy or pancreatic divisum[13]. While genetic testing to rule out hereditary pancreatitis is a consideration[14], this was omitted in our modified Naranjo score as none of the included articles performed genetic tests for confirming/excluding hereditary pancreatitis. Additionally, there are no strict recommendations on the exact indications for genetic counselling and/or testing in AP, limiting its utility for inclusion in our proposed scoring system[15].

Data extraction

Three authors (Tang ZJT, Chua BQY, and Lim PK) performed all data extraction independently using the systematic review management tool Covidence (https://www.covidence.org). The following data were extracted: Year of study, age, and gender of the patient, and features relevant to the modified Naranjo score, as shown in Table 1.

RESULTS

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67

Table 1 Modified Naranjo Score used to grade the included studies with respective points allocated to each criterion

Criteria	Yes	No	Unsure
Are there published reports of the COVID-19 causing acute pancreatitis?	+ 1	0	0
Was there short latency (≤ 7 d) between the onset of infection and the diagnosis of acute pancreatitis?	+ 2	- 1	0
Was there a temporal relationship (≤ 1 mo) between onset of infection and onset of acute pancreatitis symptoms?	+ 1	- 1	0
Did the acute pancreatitis resolve following resolution of the infection?	+ 1	- 1	0
Were all commonly known causes of acute pancreatitis ruled out? (*e.g.*, gallstones/choledocholithiasis, alcohol, hypertriglyceridaemia, hypercalcaemia, ERCP, trauma)	+ 1	- 1	0
Was a serum IgG4 level checked? (To rule out autoimmune pancreatitis)	+ 1	0	0
Does the patient have or was the patient recently diagnosed with an infection (other than COVID-19) which could cause pancreatitis?	- 1	+ 1	0
Was an EUS and/or MRCP performed? (*e.g.*, to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum)	+ 1	- 1	0

COVID-19: Coronavirus disease 2019; IgG4: Immunoglobulin G4; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; MRCP: Magnetic resonance cholangiopancreatography.

articles were included in the final analysis (Figure 1). There was a total of 76 patients which had AP reported to be due to COVID-19. Data extracted from all cases were reported in Table 2[16-82]. Seventy-six patients with a mean age of 47.8 years (range 18-94) were reported. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP (Figure 2A). Majority of patients (73.7%, n = 56/76) had a short duration of latency (≤ 7 d) between the onset of infection and AP. Most reports did not explore the temporal relation between COVID-19 and AP; only 20 (26.3%) patients were described to have a temporal relationship between COVID-19 and AP. Common aetiologies of AP were ruled out in 45 (59.2%) patients. Serum IgG4 Levels were tested to rule out autoimmune pancreatitis in 9 (13.6%) patients. None of the patients were recently diagnosed with an infection known to cause AP, *e.g.*, Coxsackie virus, cytomegalovirus, or herpes simplex virus. Only a minority (6.6%, n = 5/76) underwent either EUS or ERCP. None of the patients underwent genetic testing, *e.g.*, *SPINK-1*, to rule out hereditary AP.

DISCUSSION

AP is a common cause of acute abdominal pain and hospital admissions and present as a disease spectrum[83]. In view of the COVID-19 pandemic, many retrospective case reports suggesting COVID-19 as a cause of AP have been published. It is an obligation to critically appraise these reports to define the strength of the association and evaluate if the co-occurrence is a mere association or actual causation. This novel study adopted the modified Naranjo scale and concluded the cause-effect relationship between COVID-19 and AP in most reports as doubtful (42.1%) or possible (51.3%).

While gallstones and alcohol consumption remain the most common aetiologies, in rare situations, viral infections have been reported to cause AP[3,4]. However, this should be a diagnosis of exclusion after ruling out of common aetiologies for AP. In our review, the reason why strong conclusions could not be drawn for most cases was the absence of serum IgG4 Levels (86.4%) to rule out autoimmune AP (score of + 1) and EUS or MRCP (93.4%) to rule out structural causes (*e.g.* occult microlithiasis or pancreatic divisum) (score of +1). Authors reporting further cases of AP where COVID-19 is suggested as an aetiology should perform these tests to rule out other aetiologies of AP prior to conclusion of COVID-19-induced AP. While the results do not confirm AP as a possible aetiology of COVID-19, it is still prudent to consider COVID-19 when patients present with AP, especially if there are concurrent clinical features of COVID-19 with temporal relation of symptoms and if other common causes are ruled out.

The current literature is torn on the idea that the SARS-CoV2 virus could have induced the recent cases of AP, where some propose a direct causal relationship and others claim the SARS-CoV2 virus as a bystander in the instances of idiopathic AP[84-86]. For the former, two possible mechanisms have been described: The direct effect of the SARS-CoV2 RNA on pancreatic tissue due to viral tropism and the indirect effect due to microthrombi formation. Regarding the direct mechanism, the expression of the angiotensin converting enzyme 2 (ACE2) protein on the SARS-CoV2 virus primes its entry into the pancreas. This expression of the ACE2 protein was similarly found in the islet and exocrine tissue microvasculature and in a subset of pancreatic ducts, as well as the TMPRSS2 proteins found in the ductal cells[84]; this suggests entry of SARS-CoV2 into pancreatic tissue resulting in AP. Regarding the

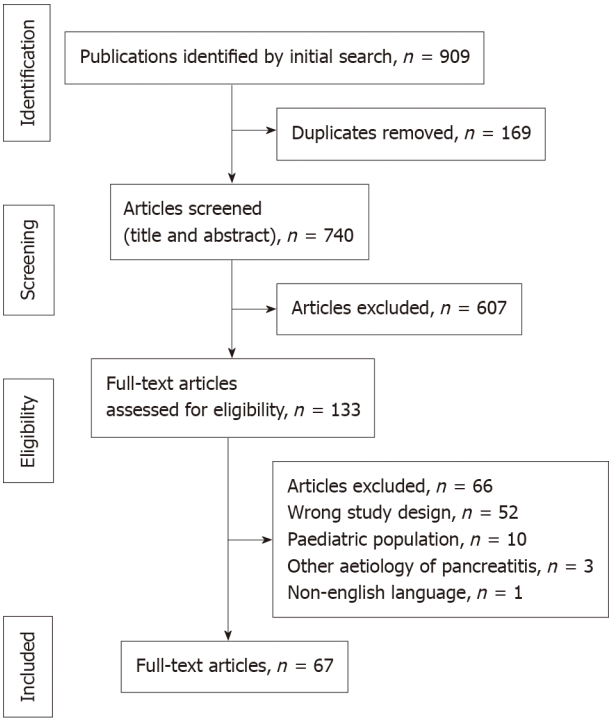
Table 2 Summary of all the included case reports (*n* = 76 patients) with respective patient demographics, modified Naranjo Score and interpretation

No	First author	Year	Patient age/Sex	Scoring								Summed score	Result[1]
1	Acherjya *et al*[16]	2020	57/F	1	2	1	1	1	0	1	-1	6	Possible
2	Al-Douri *et al*[17]	2020	45/F	1	2	1	0	1	0	1	-1	5	Possible
3	Al-Harmi *et al*[18]	2021	52/F	1	0	1	0	1	0	1	-1	3	Doubtful
4	Ali *et al*[19]	2021	53/M	1	0	1	0	-1	0	1	-1	1	Doubtful
5	Aloysius *et al*[20]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
6	Alves *et al*[21]	2020	56/F	1	2	1	1	1	0	1	1	8	Probable
7	Alwaeli *et al*[22]	2020	30/M	1	2	1	0	1	0	1	-1	5	Possible
8	Amé and Balderramo[23]	2022	42/F	1	2	1	0	1	0	1	-1	5	Possible
9			65/F	1	0	1	0	1	1	1	1	6	Possible
10	Anand *et al*[24]	2020	59/F	1	0	1	0	1	0	1	-1	3	Doubtful
11	Arjun *et al*[25]	2020	34/M	1	2	1	1	1	1	1	-1	7	Probable
12	Bains *et al*[26]	2020	68/M	1	2	1	0	-1	0	1	1	5	Possible
13	Bokhari and Mahmood[27]	2020	32/M	1	2	1	0	1	0	1	-1	5	Possible
14	Bouali *et al*[28]	2021	60/F	1	2	1	0	-1	0	1	-1	3	Doubtful
15	Brikman *et al*[29]	2020	61/M	1	0	1	0	1	0	1	-1	3	Doubtful
16	Canastar *et al*[30]	2020	64/M	1	2	1	0	-1	0	1	-1	3	Doubtful
17	Chandra *et al*[31]	2021	53/M	1	2	1	0	-1	0	1	-1	3	Doubtful
18	Cheung *et al*[32]	2020	38/M	1	2	1	0	1	1	1	1	8	Probable
19	Chivato *et al*[33]	2021	55/M	1	2	1	1	1	0	1	1	8	Probable
20	Dufani *et al*[34]	2020	27/F	1	2	1	0	1	0	1	-1	5	Possible
21	Elhence *et al*[35]	2020	31/F	1	0	1	0	-1	0	1	-1	1	Doubtful
22			40/M	1	0	1	0	-1	0	1	-1	1	Doubtful
23			42/M	1	0	1	0	-1	0	1	-1	1	Doubtful
24	Fernandes *et al*[36]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
25	Fiore *et al*[37]	2021	42/M	1	2	1	0	1	0	1	-1	5	Possible
26			70/M	1	0	1	0	1	0	1	-1	3	Doubtful
27	Gadiparthi *et al*[38]	2020	40/M	1	2	1	1	-1	0	1	-1	4	Possible
28	Gadiparthi *et al*[39]	2021	74/F	1	2	1	0	1	0	1	-1	5	Possible
29	Gonzalo-Voltas *et al*[40]	2020	76/F	1	2	1	1	1	0	1	-1	6	Possible
30	Gupta *et al*[41]	2021	25/F	1	2	1	1	1	0	1	-1	6	Possible
31	Hadi *et al*[42]	2020	47/F	1	2	1	0	-1	0	1	-1	3	Doubtful
32			68/F	1	2	1	0	-1	0	1	-1	3	Doubtful
33	Hanif *et al*[43]	2021	30/F	1	2	1	0	-1	0	1	-1	3	Doubtful
34	Hassani *et al*[44]	2020	78/F	1	2	1	0	1	0	1	-1	5	Possible
35	Ibrahim *et al*[45]	2020	33/M	1	2	1	0	-1	0	1	-1	3	Doubtful
36	Jespersen Nizamic *et al*[46]	2020	49/W	1	0	1	0	1	0	1	-1	3	Doubtful
37	Kandasamy[47]	2020	45/F	1	2	1	1	1	0	1	-1	6	Possible
38	Karimzadeh *et al*[48]	2020	65/F	1	2	1	0	-1	0	1	-1	3	Doubtful
39	Kataria and Sharif[49]	2020	49/F	1	2	1	1	1	0	1	-1	6	Possible
40	Kolhe *et al*[50]	2020	19/F	1	2	1	1	1	0	1	-1	6	Possible

41	Kumaran *et al*[51]	2020	67/F	1	2	1	1	1	1	1	-1	7	Probable
42	Kurihara *et al*[52]	2020	55/M	1	0	1	0	1	0	1	-1	3	Doubtful
43	Lakshmanan and Malik[53]	2020	68/M	1	2	1	0	1	0	1	-1	5	Possible
44	Maalouf *et al*[54]	2021	62/M	1	2	1	0	1	0	1	-1	6	Possible
45	Mazrouei *et al*[55]	2020	24/M	1	2	1	0	-1	0	1	-1	3	Doubtful
46	Meireles *et al*[56]	2020	36/F	1	0	1	1	1	1	1	-1	5	Possible
47	Merza *et al*[57]	2020	57/M	1	2	1	0	-1	0	1	-1	3	Doubtful
48			70/M	1	0	1	0	-1	0	1	-1	1	Doubtful
49	Meyers *et al*[58]	2020	67/M	1	2	1	0	-1	1	1	-1	4	Possible
50	Miao *et al*[59]	2020	26/F	1	2	1	0	-1	0	1	-1	3	Doubtful
51	Mobin *et al*[60]	2020	18/M	1	2	1	0	1	0	1	-1	5	Possible
52			66/M	1	2	1	1	-1	0	1	-1	4	Possible
53	Mohammadi Arbati and Molseghi [61]	2021	28/M	1	2	1	0	-1	0	1	-1	3	Doubtful
54	Muhammad Abrar Jeelani *et al*[62]	2021	24/M	1	0	1	1	1	0	1	-1	4	Possible
55	Naqvi *et al*[63]	2020	69/F	1	2	1	0	1	0	1	-1	5	Possible
56	Narang *et al*[64]	2021	20/F	1	2	1	0	1	1	1	-1	6	Possible
57	Patnaik *et al*[65]	2020	29/M	1	2	1	0	1	0	1	-1	5	Possible
58	Pinte and Baicus[66]	2020	47/M	1	0	1	0	1	0	1	-1	4	Possible
59	Purayil *et al*[67]	2020	58/M	1	2	1	0	-1	0	1	-1	3	Doubtful
60	Rabice *et al*[68]	2020	36/F	1	2	1	1	1	0	1	-1	6	Possible
61	Rotar *et al*[69]	2020	39/M	1	2	1	0	-1	0	1	-1	3	Doubtful
62	Sandhu *et al*[70]	2021	25/F	1	2	1	0	1	0	1	-1	5	Possible
63	Shinohara *et al*[71]	2020	58/M	1	0	1	1	-1	0	1	-1	2	Doubtful
64	Simou *et al*[72]	2020	67/NM	1	0	1	0	-1	0	1	-1	1	Doubtful
65	Singh and Kharoud[73]	2020	94/F	1	2	1	0	1	1	1	-1	6	Possible
66			58/F	1	0	1	0	1	1	1	-1	4	Possible
67	Srinivasan *et al*[74]	2021	52/F	1	2	1	0	-1	0	1	-1	3	Doubtful
68	Tollard *et al*[75]	2021	32/F	1	2	1	0	1	0	1	-1	5	Possible
69	Tomasi *et al*[76]	2021	31/M	1	2	1	0	1	0	1	-1	5	Possible
70	Truscello *et al*[77]	2020	71/M	1	2	1	0	-1	0	1	-1	3	Doubtful
71	Wang *et al*[78]	2020	42/M	1	2	1	0	1	0	1	-1	5	Possible
72			35/M	1	2	1	1	1	0	1	-1	6	Possible
73	Wifi *et al*[79]	2021	72/F	1	2	1	0	-1	0	1	-1	3	Doubtful
74	Yamamoto *et al*[80]	2021	70/F	1	0	1	0	-1	0	1	-1	1	Doubtful
75	Zeng *et al*[81]	2020	36/M	1	2	1	0	0	0	1	-1	4	Possible
76	Zielecki *et al*[82]	2020	38/M	1	0	1	1	-1	0	1	0	3	Doubtful

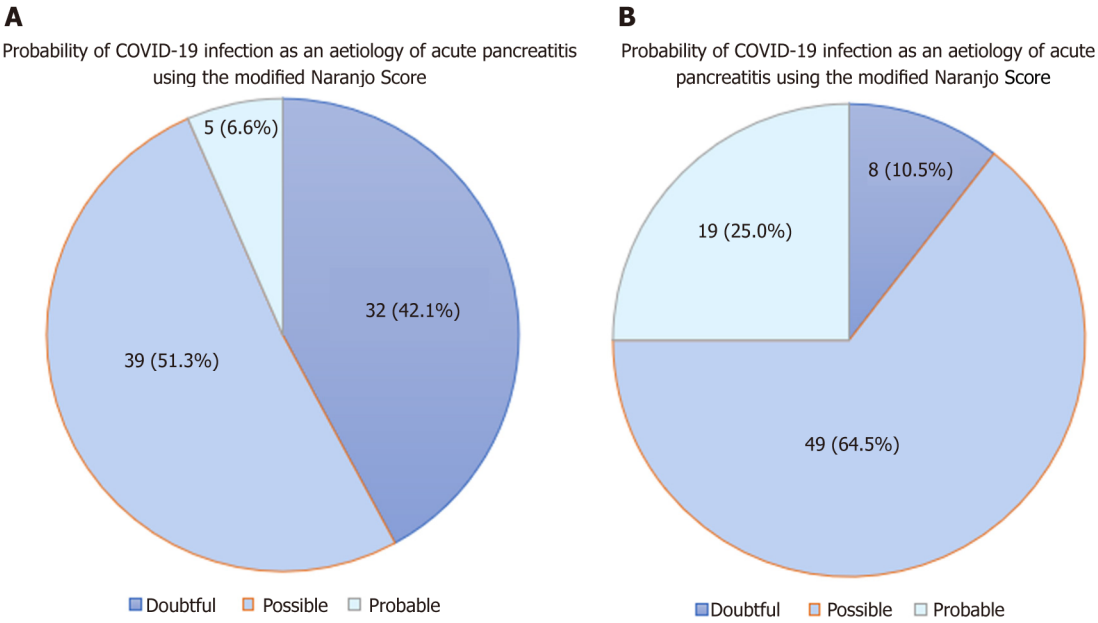
[1]Interpretation of the modified Naranjo score is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable.
F: Female; M: Male; NM: Not mentioned.

indirect mechanism, SARS-CoV2 causes microthrombi formation due to its ability to invade endothelial cells *via* the ACE2 protein. This phenomenon is also observed in other virus infections, such as viral hepatitis[85]. Microthrombus in vasculature triggers hypoperfusion of the pancreas, resulting in ischemic pancreatitis. However, the above theories are merely conjectures made from parallel existing conditions superimposed on an under-researched virus. The possibility of COVID-19 being a pure bystander in the formation of AP also exists. This may be a situation of Occam's razor– where we look



DOI: 10.3748/wjg.v29.i13.2050

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses figure showing the study selection process.



DOI: 10.3748/wjg.v29.i13.2050

Figure 2 Graphical representation of the probability of coronavirus disease 2019 as an aetiology of acute pancreatitis. A: Using the modified Naranjo score proposed; B: Using the modified Naranjo score, if a score of 1 was subtracted from the minimum cut-off for each category, where ≤ 2 is for doubtful, 3-5 is for possible and ≥ 6 is for probable significance. COVID-19: Coronavirus disease 2019.

to connect the dots between a relatively new disease and the patients' manifestations– *vs* a pure case of Hickam's dictum– where a patient happens to present with two mutually exclusive conditions. After all, it must be noted that idiopathic AP is a function of diagnostic workup efforts[86]. The lack of serological tests for IgG4, MRCP scan, and EUS imaging could inflate the association between COVID-19 and AP. As randomized studies cannot be conducted to establish COVID-19 as an aetiology for AP, critical appraisal of retrospective data is essential to discern association from causation.

The temporal relation of events is one of the important determinants of distinguishing causation from the association. Many authors did not explicitly mention a temporal relation of onset or resolution of COVID-19 and AP. While COVID-19 infections primarily involve the respiratory system, multi-systemic involvement have been reported. Since COVID-19 is a prerequisite for COVID-19 associated AP, we shall discuss the respiratory and imaging features first. COVID-19-induced AP may manifest in the absence of any respiratory symptoms or radiological evidence of lung involvement. Purayil *et al*[67] reported a 58-year-old male presenting with abdominal pain; a polymerase chain reaction (PCR) for COVID-19 was only done in response to his chest x-ray, which revealed bilateral infiltrates in the absence of respiratory symptoms[67]. A chest x-ray is routinely performed in patients with acute abdomen to rule out free air under the diaphragm secondary to hollow viscus perforation, which also can cause hyperamylasaemia and remain an important differential diagnosis of AP[87]. Additionally, chest x-ray findings form a part of scoring systems to predict severity and clinical outcomes in AP patients[88,89]. In the patient reported by Purayil *et al*[67], the chest x-ray was sensitive to detect COVID-19 changes and complemented the diagnostic work-up for epigastric pain. As most patients with AP will have a chest x-ray performed and due to the widespread prevalence of COVID-19 in the community, it is not possible to ascertain if COVID-19 resulted in AP or if the two diseases merely occurred simultaneously yet independently of each other.

The prevalence of gastrointestinal symptoms in COVID-19 infected patients ranges from 3.0%-79%, of which only 2.2%-6.0% of patients present with abdominal pain[90-92]. Abdominal pain is the most common symptom of AP and acute epigastric pain is one of the key diagnostic criteria of AP. Thus it remains unclear if gastrointestinal manifestations of COVID-19 are secondary to AP. Though resuscitation and early management of AP patients are not determined by aetiology, definite management for prevention of future occurences is determined by aetiology. Thus, knowledge of COVID-19 as an aetiology of AP is important as it stops the pursuit of aetiology identification, guides physician on the counselling of their patients, and impacts management decision for cholecystectomy. In patients with mild biliary pancreatitis, index admission laparoscopic cholecystectomy is considered good clinical practice while surgery should be delayed in patients with COVID-19 infection to minimize risk to healthcare workers and reduce patient morbidity[93,94]. Shao *et al*[95] analysed 589 patients with COVID-19 infection prior to surgery and concluded that postoperative mortality was nearly 6 times higher for patients infected with COVID-19 within 2 wk before surgery when adjusting for patient and procedure level factors[95]. Furthermore, delaying cholecystectomy in patients with biliary pancreatitis could increase the risk of future biliary events[88]. Thus, determining the aetiology of AP is essential as it impacts clinical decisions.

In addition to abdominal pain and imaging features, it is important to discuss the role of serum enzymes. While both amylase and lipase are usually measured in patients presenting with acute abdomen suggesting AP, the timing at which they rise can be cross-referenced to the time at which COVID-19 infection was diagnosed, which may shed some light on whether COVID-19 is a cause of AP, or merely coincidental. Amylase tends to rise within 3 to 6 h of AP and persists for up to 5 d. However, serum amylase has a relatively short half-life of 12 h and may return to normal limits within a day. Serum lipase rises similarly within 3 to 6 h, peaks at 24 h, and persists for 8 to 14 d. Hence, a patient with raised amylase or lipase alongside a concomitant COVID-19 infection before this time span of 12-24 h may still have AP caused by COVID-19. This clarity blurs if we consider the virus's incubation period and heterogeneity in the clinical presentation of both COVID-19 and AP. Stephens *et al*[96] reported 234 COVID-19 positive patients in the critical care unit, of which 52 (22.2%) patients had peak amylase three times the upper limit of normal, of which only 4 (1.7%) met the revised Atlanta criteria for diagnosis of AP[96]. Furthermore, some authors report COVID-19 associated hyperamylasemia secondary to pancreatic injury which does not amount to clinical AP[97,98]. This could be attributed to amylase release from other viscera like the gastrointestinal tract or elevated serum levels due to reduced renal excretion as a result of critical illness-related kidney injury[96]. A contrast-enhanced imaging remains an essential tool to diagnose AP, but it would not establish COVID-19 as a causative aetiology.

As the temporal occurrence of events (*e.g.* onset of abdominal pain, chest x-ray imaging, serum enzymes, and abdominal imaging) do not aid the distinction of causation or association, a different approach is essential. This situation is similar to the dilemma of attributing a clinical presentation towards an adverse drug reaction. The Naranjo score is a useful tool that aids clinical judgment in differentiating the cause-effect relationship between a drug and its possible adverse drug reaction[9]. While useful in determining the causality, it should be noted that the scale does not offer prognostic information. In addition, the Naranjo score includes isolating toxic concentrations of drugs in body fluids, clinical response to placebo administration, and a drug rechallenge to assess symptoms occurrence. While most authors report positive COVID-19 rapid antigen test and PCR, the response to placebo and re-infection are not reported. It is not possible to assess if the patients were re-infected with COVID-19 (after reporting of the case) with repeat AP and unethical to do this experimentally. Thus, no comment can be made on whether a 're-challenge' would bring about similar symptoms. If these parameters are scored negative, many cases would be disadvantaged from the strength of causation, and thus, a modification of the Naranjo score that excludes placebo administration and re-infection was considered essential to determine COVID-19 as a cause of AP fairly. This approach has been reported in the past for sulphasalazine-induced AP and prednisolone induced pneumatosis coli[10,99]. Using this

strategy, we determined the strength of association and possible causation as doubtful, possible or probable. Other established methods of determining a causal relationship between various aetiological agents and AP include the Badalov categorisation[100]. The Badalov categorisation was similarly designed to investigate association between drugs and adverse drug reactions; this involves assigning drugs into 5 categories (Class Ia, Ib, II, III and IV) based on the number of case reports published, drug rechallenge, latency and whether alternative causes were excluded. However, we have chosen to adopt the Naranjo score as it allows for a case-by-case evaluation of each report as opposed to a blanket categorization of COVID-19 as a possible aetiological agent of AP. Secondly, the Naranjo score also provides a better idea on the degree of association by generating data on a numerical scale which allows for in-depth analysis. Centers should continue reporting such occurrences of COVID-19-induced pancreatitis and consider incorporating our modified Naranjo score; artificial intelligence methods may subsequently be used to diagnose COVID-19-induced pancreatitis[101,102].

However, our study has its limitations. Firstly, given the nature of our study, prospective studies and systematic reviews were not analysed as they lack individual patient data. Additionally, interpretation of this study is limited by the small sample size of 76 patients. Secondly, a majority of included case reports date after the introduction of the COVID-19 vaccine. Many reports did not mention the vaccination status of the patient; COVID-19 vaccination has also been reported as a potential cause of AP which adds on to the dilemma[24,103]. Thirdly, with the advent of novel drugs used in COVID-19 treatment, the possibility of drug-induced AP needs to be considered as a differential diagnosis. Remdesivir is an antiviral drug widely used in the management of COVID-19 and it is increasingly reported to cause pancreatic injury with associated hyperamylasaemia as well as AP[104,105]. Fourthly, authors may not have reported cases where patients were reinfected by COVID-19. This is a potential limitation in the calculation of the modified Naranjo score. Fifthly, the determination of the criteria for "doubtful", "possible" and "probable" is arbitrary. If we subtract one point from the minimum of each category (*i.e.* ≤ 2 for doubtful, 3-5 for possible and ≥ 6 for probable) to increase the causation strength, this would result in 8 (compared to 32) patients, 49 patients (compared to 39), and 19 patients (compared to 5) respectively (Figure 2A and B). As the median score was 4, if we use it as the cut-off, 32 patients (score < 4) will be categorized as "less likely" and 44 patients (score ≥ 4) as "more likely" to have COVID-19-induced AP. Thus, the assignment of scores, though partly arbitrary, the fact still prevails that existing reports have doubt about COVID-19 infection causing AP. Lastly, while some of the screened cases reported fatalities[13], our study did not assess the severity and mortality of COVID-19 induced AP.

CONCLUSION

The use of our proposed modified Naranjo score may help to determine whether COVID-19 is a likely aetiology of AP and may assist clinicians in making useful clinical decisions. The current evidence is weak to establish a strong causal link between COVID-19 and AP, and more evidence is necessary before COVID-19 should be incorporated as a "textbook aetiology" of AP.

ARTICLE HIGHLIGHTS

Research background

Acute pancreatitis (AP) is a disease spectrum ranging from mild to severe disease. During the coronavirus disease 2019 (COVID-19) pandemic, numerous reports of AP have been published, with most authors concluding a causal relationship between COVID-19 and AP.

Research motivation

Published reports or case series of COVID-19-induced AP are retrospective in nature and are unable to accurately determine the cause-effect relationship between COVID-19 and AP.

Research objectives

This study aims to establish whether COVID-19 is a cause of AP by proposing a scoring system *i.e.* the modified Naranjo scoring system.

Research methods

A systematic review was conducted on PubMed, World of Science and Embase for articles reporting COVID-19 and AP from inception to August 2021. Exclusion criteria were cases of AP which were not reported to be due to COVID-19 infection, age < 18 years old, review articles and retrospective cohort studies. The original 10-item Naranjo scoring system (total score 13) was devised to approximate the likelihood of a clinical presentation to be secondary to an adverse drug reaction. We modified the

original scoring system into a 8-item modified Naranjo scoring system (total score 9) to determine the cause-effect relationship between COVID-19 and AP. A cumulative score was decided for each case presented in the included articles. Interpretation of the modified Naranjo scoring system is as follows: ≤ 3: Doubtful, 4-6: Possible, ≥ 7: Probable cause.

Research results

The initial search resulted in 909 articles, with 740 articles after removal of duplicates. A total of 67 articles were included in the final analysis, with 76 patients which had AP reported to be due to COVID-19. The mean age was 47.8 (range 18-94) years. Majority of patients (73.3%) had ≤ 7 d between onset of COVID-19 infection and diagnosis of AP. There were only 45 (59.2%) patients who had adequate investigations to rule out common aetiologies (gallstones, choledocholithiasis, alcohol, hypertriglyceridemia, hypercalcemia and trauma) of AP. Immunoglobulin G4 testing was conducted in 9 (13.5%) patients to rule out autoimmune AP. Only 5 (6.6%) patients underwent endoscopic ultrasound and/or magnetic resonance cholangiopancreatogram to rule out occult microlithiasis, pancreatic malignancy and pancreas divisum. None of the patients had other recently diagnosed viral infections apart from COVID-19 infection, or underwent genetic testing to rule out hereditary AP. There were 32 (42.1%), 39 (51.3%) and 5 (6.6%) patients with doubtful, possible, and probable cause-effect relationship respectively between COVID-19 and AP.

Research conclusions

Current evidence is weak to establish a strong link between COVID-19 and AP. Investigations should be performed to rule out other causes of AP before establishing COVID-19 as an aetiology.

Research perspectives

The use of our proposed modified Naranjo score may help to determine whether COVID-19 is a likely etiology of AP and may assist clinicians in making useful clinical decisions.

FOOTNOTES

Author contributions: Teng TZJ, Chua BQY and Lim PK contributed equally to this work; Teng TZJ, Chua BQY and Lim PK performed the literature search, data collection and wrote the manuscript; Chan KS wrote the manuscript and revised the manuscript; Shelat VG conceptualised the study design and idea, and revised the manuscript; All authors have read and approve the final manuscript.

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