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## Dietary Lectin exclusion: The next big food trend?

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### Abstract

Until recently, with the exception of coeliac disease, gastroenterologists have not been particularly interested in the role of diet in the management of gastrointestinal disorders. However, patients have always felt that diet must play a part in their symptoms and, in the absence of any medical interest, have turned to alternative dietary practitioners for help, which can often have no evidence base. Fortunately, with the advent of the FODMAP diet (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) and the realisation that diet can have a profound effect on the microbiome, medical opinion is now changing. Nevertheless, research on the various diets that are now available is often completely lacking. Lectins are carbohydrate binding proteins which are widely distributed in nature and are found in a whole variety of commonly consumed foods. It seems likely that the exclusion of lectins from the diet could become the next "food fashion" for alternative practitioners to promote, especially as there is some evidence to suggest that certain lectins may be harmful to health. It is, therefore, the purpose of this viewpoint to try and stimulate research on the dietary effects of lectins, which is currently minimal, so that we can pre-empt a situation where we are unable to give patients or the public evidence based advice on this topic.

**Key words:** Dietary lectins; Exclusion diets; Gastrointestinal system; Harm; Carbohydrate

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**Core tip:** Patients with gastrointestinal problems, as well as the general public, are being offered an increasing number of different diets which claim to improve their health, often without any evidence to support a beneficial effect. Lectins are carbohydrate binding proteins which are found in many foods and some of them, such as those found in red kidney beans, can cause gastrointestinal symptoms if not cooked properly. Consequently,

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it is possible that a lectin exclusion diet could become fashionable in the future and research is needed to find out under what circumstances, if any, such a diet may be advisable.

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## INTRODUCTION

The majority of patients with gastrointestinal problems, especially those of a functional nature, consider that diet is important either in the cause of their symptoms or at least in their exacerbation. Unfortunately, until relatively recently the medical profession has largely ignored the role of diet in gastroenterology other than to advise patients to eat more fibre. However, in 1994 we showed that cereal fibre can actually exacerbate the symptoms of irritable bowel syndrome (IBS)<sup>[1]</sup>. In addition, we also found that fruit and vegetables could cause problems and assumed that this was likely to be as a result of their fibre content<sup>[1]</sup>. Despite these findings being published in the *Lancet*, this lack of interest in the contribution of diet to gastrointestinal health persisted with the void gradually being filled by alternative dietary practitioners as well as the marketing of a whole variety of tests for the detection of food allergies and intolerances. The proliferation of these alternative sources of advice coupled with the advent of the Internet may also partly explain why even healthy members of the general public have now become interested in the possible harmful effects of some dietary components. As a result of this, a bewildering array of diets are now fashionable.

## GLUTEN

The exclusion of gluten is attracting particular attention. Obviously, the role of gluten in coeliac disease is beyond doubt but there is now interest in the possible effects of gluten in those individuals with the genetic predisposition to coeliac disease and even concepts such as non-coeliac gluten sensitivity<sup>[2]</sup>. Furthermore, large numbers of apparently healthy individuals are now adopting a gluten free diet<sup>[3]</sup>. Consequently, it is absolutely essential that the medical profession start undertaking good quality research on the role of gluten and other dietary components in health and disease so that the public can be given evidence based advice about their diet rather than having to trawl through questionable information on the Internet.

## FODMAPs

Fortunately, the advent of the low FODMAP diet (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) for the treatment of IBS has at last galvanised interest in dietary interventions amongst the gastroenterology community and, hopefully, this will stimulate further research on other dietary interventions in both gastroenterology as well as other specialties. FODMAPs are carbohydrates that are poorly absorbed by the gut and are, therefore, fermented by bacteria. This leads to symptoms, especially in patients with IBS, possibly by exacerbating the gut hypersensitivity that exists in these patients<sup>[4]</sup>. It seems likely that the detrimental effect of fruit and vegetables on symptoms of IBS that we previously reported is more likely to be due to FODMAPs rather than fibre as we surmised, although a dual effect may be a possibility. FODMAPs are contained in cereals, fruits, vegetables, and legumes as well as milk products and there is now reasonably good evidence that FODMAP restriction in IBS can improve symptoms<sup>[5]</sup>. However, there are some concerns about whether this diet can lead to changes in the gut microbiota that may not be entirely desirable<sup>[6]</sup>. Given the sometimes dramatic reduction in symptoms that can be seen in patients on a low FODMAP diet it is possible that, in the future, it might be tempting for even healthy individuals to experiment with this approach.

## LECTINS

Lectins are carbohydrate binding proteins that are widely distributed in nature and occur in a variety of foods such as cereals, fruit, vegetables, animal products and fish<sup>[6]</sup>. There is no universally accepted classification of lectins but they do have characteristics that differ from other proteins. Some lectins such as ricin, which is derived from the castor bean, are highly toxic with the ingestion of even miniscule quantities of ricin proving fatal whereas other lectins are relatively harmless<sup>[7]</sup>. Lectins, such as phytohaemagglutinin and concanavalin A, agglutinate red cells and act as lymphocyte mitogens with these properties having been used in the laboratory for many years. Much of the research on the results of consuming lectins is relatively old and largely confined to the effect of plant lectins on animals where, for instance, they can survive transit through the gut and have a variety of local and systemic effects<sup>[8-10]</sup>. Furthermore, in contrast to animal proteins, lectins are resistant to heat and even cooking can fail to inactivate them unless it is above 100 °C for as long as thirty minutes or more<sup>[11]</sup>. Animal studies have shown that lectins, which have an affinity for gut epithelium, can interfere with absorption of nutrients with these effects sometimes being called “anti-nutritional”<sup>[12,13]</sup>. For instance, phytohaemagglutinin, which is found in high concentrations in the red kidney bean, has a range of effects on the gut including decreased acid secretion, crypt hyperplasia, changes in the brush border and even an indirect effect on the pancreas and these effects on the gut are not confined to this particular lectin<sup>[10,14-16]</sup>. There is also evidence that some lectins may affect the gut microbiota as well as having systemic effects such as the modulation of inflammation and immune function<sup>[17,18]</sup>. It should be noted that these latter properties may not necessarily always be negative indicating that the therapeutic potential of some of these proteins might also be worth exploring.

Despite this evidence of the detrimental effects of lectins in animals, their potential to cause harm in humans has received surprisingly little scientific attention although “food poisoning” due to red kidney bean consumption has been reasonably well documented<sup>[19]</sup>. However, much more needs to be known about which lectins are harmful and the effects of dose and duration of consumption. It is also interesting to note that many of the foods that are excluded in the low FODMAP diet are those that also contain lectins. This raises the possibility that it may not just be the FODMAPs that are causing problems in those who benefit from their exclusion.

## CONCLUSION

As a result of their potential for toxicity and their “anti-nutritional effects” it is almost inevitable that lectin exclusion could well become a big food fad<sup>[13]</sup>. Consequently, now is the time to resume research on this ubiquitous family of proteins so that we fully understand their role in health and disease. This would then enable us to advise our patients and the general public accordingly, rather than having to play “catch up” after everybody starts wondering whether they should be excluding some or adding others to their diet.

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## Immunotherapy for hepatocellular carcinoma: Current and future

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### Abstract

Hepatocellular carcinoma (HCC) arises on the background of chronic liver disease. Despite the development of effective anti-viral therapeutics HCC is continuing to rise, in part driven by the epidemic of non-alcoholic fatty liver disease. Many patients present with advanced disease out with the criteria for transplant, resection or even locoregional therapy. Currently available therapeutics for HCC are effective in a small minority of individuals. However, there has been a major global interest in immunotherapies for cancer and although HCC has lagged behind other cancers, great opportunities now exist for treating HCC with newer and more sophisticated agents. Whilst checkpoint inhibitors are at the forefront of this revolution, other therapeutics such as inhibitory cytokine blockade, oncolytic viruses, adoptive cellular therapies and vaccines are emerging. Broadly these may be categorized as either boosting existing immune response or stimulating de novo immune response. Although some of these agents have shown promising results as monotherapy in early phase trials it may well be that their future role will be as combination therapy, either in combination with one another or in combination with treatment modalities such as locoregional therapy. Together these agents are likely to generate new and exciting opportunities for treating HCC, which are summarized in this review.

**Key words:** Adoptive cell therapy; Cancer vaccine; Checkpoint inhibitor; Hepatocellular carcinoma; Immunotherapy; Liver cancer; Oncolytic virus

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**Core tip:** A significant proportion of patients with hepatocellular carcinoma (HCC) present with advanced disease, for which there are limited systemic therapeutic options.

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Complicating this, HCC often develops on a background of cirrhosis, which can preclude the use of certain cytotoxic agents. Immunotherapy has previously not been an available therapeutic option in HCC. However, checkpoint inhibition therapy was recently licensed as a second line option for advanced disease. Multiple other promising agents are in development which boost existing immune response or stimulate a *de novo* immune response. These agents are discussed herein.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer, constituting 75%-85% of cases. It presents a significant health burden as the sixth most commonly diagnosed cancer worldwide in 2018. In addition, reflecting its poor outcome, it was the fourth leading cause of cancer death<sup>[1]</sup>. The incidence of HCC varies country by country depending on the relative prevalence of key risk factors. These include chronic infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV), as well as aflatoxin exposure<sup>[2]</sup>. These are more common in lower human development index countries. Although vaccination against HBV is recommended to reduce HCC development and has been used successfully in countries such as Taiwan, problems such as logistics of delivery and vaccine availability are significant factors which limit this approach<sup>[3]</sup>.

HCV and HBV related cirrhosis are associated with the highest incidences of HCC. However, other aetiologies of cirrhosis, including non-alcoholic fatty liver disease (NAFLD), alcohol related liver disease and hereditary haemochromatosis are also strongly associated with an increased incidence of HCC<sup>[4]</sup>. Furthermore, any optimism about the revelatory impact new HCV drugs will have on HCC burden is forestalled by the global rise in NAFLD, type 2 diabetes mellitus and the “metabolic syndrome” as endemic risk factors for HCC development<sup>[5,6]</sup>. In particular the incidence of HCC is rising, particularly in countries with a high socio-demographic index, and consistent with this HCC may arise on the background of a non-cirrhotic liver in NALFD<sup>[7,8]</sup>.

Surgery is the most successful treatment for HCC, either liver transplantation or liver resection depending on liver function, the presence of portal hypertension and tumour burden. Selection for surgery remains based upon Barcelona clinic liver cancer (BCLC) criteria for the most part, although “extended criteria” may be used by experienced centres<sup>[9]</sup>. Unfortunately given that liver cancer usually occurs on the background of cirrhosis, the residual liver post-resection still presents an environment predisposing to the development of subsequent tumours. Thus recurrence is a significant problem<sup>[10]</sup>. Transplantation obviates this concern to an extent by removing the background liver but patients may be outwith criteria for transplant at presentation or subsequently become ineligible while waiting for a suitable donor organ. Post-liver transplantation HCC recurrence appears to occur in 10%-20% of patients<sup>[11]</sup>.

Locoregional therapy is the main alternative therapy depending on the stage of the underlying liver disease. This largely comprises two major types: (1) Percutaneous ablation such as microwave ablation or radiofrequency ablation (RFA); (2) Intra-arterial chemoembolotherapy, namely transcatheter chemoembolization<sup>[9]</sup>. Ablation may even be a first line option over surgery in selected early stage tumours with comparable mortality rates, albeit higher recurrence rates<sup>[12]</sup>. Nonetheless, locoregional therapy is for the most part not curative treatment with recurrence being common.

Unfortunately the majority (> 70%) of patients present with advanced disease outwith the criteria for transplant, surgery or locoregional therapeutic options<sup>[13]</sup>. For these patients there remains a paucity of approved therapeutic options. Sorafenib is an oral multi-tyrosine kinase inhibitor (TKI), targeting a number of signaling pathways such as vascular endothelial growth factor (VEGF), and increasing median survival by 3 mo<sup>[14]</sup>. On this basis it is recommended as the standard first line systemic therapy for patients with Child-Pugh A cirrhosis and BCLC-C<sup>[9]</sup>. Another oral multi-TKI lenvatinib is now recommended as alternative first line therapy based on non-inferiority to sorafenib<sup>[9,15]</sup>. Based upon survival benefits versus placebo in patients

previously treated with sorafenib both oral multi-TKIs regorafenib and cabozantinib have been added as second line systemic therapeutic options<sup>[16,17]</sup>. Importantly, liver cirrhosis precludes the potential use of many cytotoxic drugs and so, combined with the resistance of HCC to a number of reagents, the development of prospective chemotherapy regimens has been relatively difficult<sup>[18]</sup>.

In recent years cancer immunotherapy has seen a rapid expansion in terms of the number of agents which confer a prognostic benefit by awakening the immune system to mount a response against developing cancers, with particular success in metastatic melanoma<sup>[19]</sup>. Given the paucity of therapeutic options it is therefore logical that these immunotherapeutic targets should be explored in HCC, particularly given the correlation between immunological findings and outcomes in HCC<sup>[20]</sup>. Recently, nivolumab was added as the first Food and Drug Administration (FDA) approved immunotherapy for HCC<sup>[13]</sup>. This expansion in the therapeutic armoury is a welcome one. In this article we review the basis for immunotherapy in HCC, the agents studied to date as well as potential future developments.

## LIVER IMMUNOBIOLOGY

### *Liver immunobiology*

In addition to its many metabolic functions the liver has an important immunoregulatory role. Its dual supply of arterial and portal systemic blood makes it a unique recipient for gut pathogen exposure. This anatomy is combined with a honeycomb-like vasculature of sinusoids densely laden with specialized immunocytes including macrophages (Kupffer cells), liver sinusoidal endothelial cells (LSECs), natural killer (NK) cells and innate T cells<sup>[21]</sup>.

The LSECs account for roughly 50% of the non-parenchymal cells within the liver<sup>[22]</sup>. In conjunction with Kupffer cells and dendritic cells (DCs) one of their roles is to act as antigen presenting cells as part of the hepatic reticulo-endothelial system<sup>[23]</sup>. Residing in the space of Disse between the parenchymal cells and LSECs are hepatic stellate cells which contribute an immune sentinel role in this nuanced interplay<sup>[24]</sup>. Further to the LSECs is an abundance of resident liver lymphocytes, including NK and innate T cells, which serve a number of roles including innate immune response against viruses, intracellular bacteria, tumours and parasites<sup>[25,26]</sup>. There is thus a rich effector population which needs to be responsive to pathogens, but also immunoregulatory when exposed to the non-pathogenic antigens that flood the liver *via* the portal vein. These include innocuous nutrient antigens, bacterial degradation products, damaged cells and of course pathogenic or toxic components. It is for this reason that the immune response within the liver requires such precise homeostatic control. The inherent immune tolerogenicity which the liver has developed to adapt to this unique environment of antigen exposure has been well described<sup>[23,27]</sup>. This manifest immunotolerant capacity is evident in the liver's relatively low rates of allograft rejection compared to other organ transplants<sup>[28,29]</sup>.

### *Immunobiology in chronic liver disease*

There are two aspects to immunobiology in the context of cirrhosis. One is that in cirrhosis there is an active immune-mediated inflammatory process and that as decompensation develops it becomes progressively systemic<sup>[30]</sup>. However, the precise nature of the immune activity in cirrhosis depends on the underlying liver disease. Combined with a dysregulated immune response that predisposes to infection, this has been elsewhere described as "cirrhosis-associated immune dysfunction"<sup>[31]</sup>. It is well established the predisposition to bacterial infection and this is most evident in acute-on-chronic liver failure<sup>[32,33]</sup>. Additionally, the structural damage of cirrhosis compromises reticulo-endothelial function leading to impaired immune surveillance<sup>[21]</sup>. However, immune dysregulation is also manifest in non-cirrhotic patients, with irregularities such as elevated levels of endogenous cytokines, and a pro-inflammatory environment especially in autoimmune liver disease and viral hepatitis<sup>[34]</sup>.

### *HCC immunobiology*

In the majority of cases HCC is associated with chronic liver disease and in particular cirrhosis. The underlying inflammatory process described above drives hepatocellular DNA damage, endoplasmic reticulum stress and subsequent necrosis of the hepatocyte which leads to regenerative nodular formation, dysplastic nodules and ultimately carcinoma<sup>[35]</sup>. HCV and HBV also drive an immune-mediated inflammatory response which promotes neoplastic change, the latter also mediating its carcinogenic properties *via* direct oncogenic transformation following incorporation into host cell

DNA<sup>[36]</sup>. Furthermore, once HCC has developed the tumour can be associated with a rich immune cell infiltrate. Detailed analysis of HCCs indicated that approximately 25% have high inflammatory scores, with high or moderate levels of lymphocyte infiltration<sup>[37,38]</sup>. As one might expect, tumour infiltrating lymphocytes (TILs) form a large component in solid tumours, in an attempt by the host to mediate an antitumour reaction<sup>[39]</sup>. Unfortunately this cellular response can be dysfunctional with a higher proportion of CD4+ (helper or T regulatory cells) to CD8+ cells. This promotes immune tolerance and has been shown to confer a worse prognosis<sup>[40]</sup>. Additionally the innate immune system may be attenuated as evidenced by the hypofunctionality of NK cells in HCC<sup>[41,42]</sup>.

However, although TILs can be identified, within the tumour microenvironment in cirrhosis, they often prove insufficient to control tumour growth<sup>[43]</sup>. Expansion of myeloid derived suppressor cells (MDSCs) as well as Tregs appears to further enable the evasion of tumour cells from immune detection<sup>[44]</sup>. This creates an immunosuppressive immune environment through the secretion of transforming growth factor (TGF- $\beta$ ). In addition there are multiple mechanisms of immune evasion including secretion of other immunoregulatory cytokines such as interleukin-10 (IL-10), downregulation of ligands that activate immune cells including MHC class I and NKG2D ligands and expression of ligands that directly inhibit lymphocytes, including both T cells and NK cells<sup>[45-48]</sup>. Thus HCC is a challenging environment for the immune system. Nevertheless, immunotherapy is one of the most promising avenues for future therapies.

## CURRENT AND FUTURE IMMUNOTHERAPEUTIC STRATEGIES IN HCC

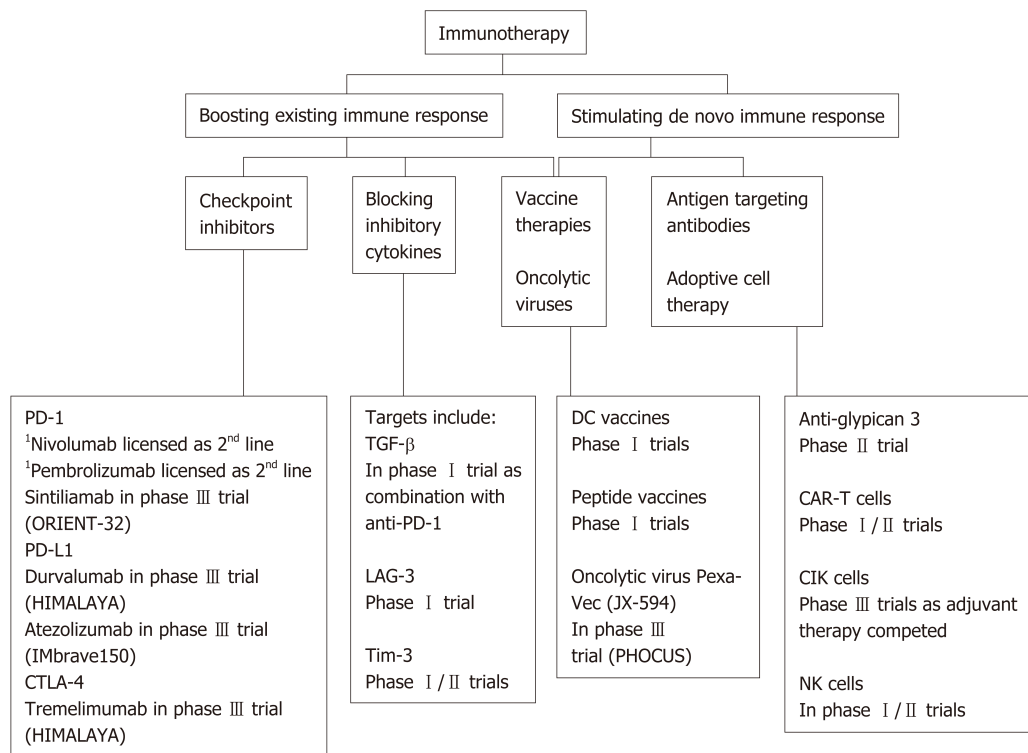
Current approaches of immunotherapy were shown in **Figure 1**. Current immunotherapeutic strategies are based on two fundamental principles: (1) The ability to unmask current immune responses; or (2) The need to stimulate new or different immune responses. Unleashing current immune response relies on there being a pre-existing immune reactivity to cancer which is being held in check by micro-environmental factors, such as inhibitory receptors on T cells especially programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), or alternatively immunosuppressive cytokines such as TGF- $\beta$ . Checkpoint inhibitors fall within this category, and importantly for these therapies to work the precise molecules that the cells are targeting do not need to be known. Conversely, antibodies that directly target molecules expressed on HCC, such as alpha-fetoprotein (AFP) or glypican-3 (GPC-3) are within the second category. These strategies can be enhanced by coupling these antibodies to effector cells, such as T cells or even NK cells. Vaccine therapeutics and the use of oncolytic viruses, discussed below, may straddle these two mechanisms by unmasking pre-existing and inducing de novo T cell responses to antigens expressed by HCC. Additionally, tumour ablation liberates antigens into the periphery and can augment CTL responses, that have been correlated with survival<sup>[49-51]</sup>.

### Checkpoint blockade

A rapidly growing list of blocking antibodies to immune checkpoints has been approved by the FDA in recent years for cancer treatment. In general, these are thought to be most effective for tumours with a high mutagenic load, such as melanoma<sup>[52]</sup>. Although these have been in trials for some time, it was not until recently that the first of these checkpoint inhibitors was approved for use in HCC, when the PD-1 inhibitor nivolumab (Opdivo®) gained FDA approval (**Table 1**).

**Programmed cell death protein 1:** PD-1 is a cell surface protein expressed on an extensive number of immune cell types, predominantly CD8+ T cells but also CD4+ T cells, B cells, NKs, Tregs, MDSCs and DCs<sup>[53-55]</sup>. It is upregulated following activation of T cells and when it binds to PD-L1 (or PD-L2) on target cells this inhibits effector T cell responses. Therefore, blocking its action is an attractive target of immunotherapy. Nivolumab's grading as an approved second line therapy for HCC is supported by evidence from the CheckMate040 trial. This was a phase I/II, open label, non-comparative, dose escalation and expansion trial in advanced HCC of mixed underlying chronic liver diseases ( $n = 262$ )<sup>[56]</sup>. 46 (96%) of 48 patients discontinued treatment in the dose escalation phase, 42 (88%) due to disease progression. However, the objective response rate was 20% in the dose-expansion phase. Incorporation of nivolumab into the AASLD guideline on HCC as second line systemic therapy was made in advance of the first phase III trial results on the basis of CheckMate040<sup>[15]</sup>.





**Figure 1 Current approaches of immunotherapy.** Summary of immunotherapeutic agents studied in hepatocellular carcinoma. CAR-T: Chimeric antigen receptor expressing T cell; CIK: Cytokine-induced killer; CTLA-4: Cytotoxic T-lymphocyte associated antigen 4; DC: Dendritic cell; LAG-3: Lymphocyte activation gene 3; NK: Natural killer; PD-1: Programmed cell death protein 1; TGF-β: Transforming growth factor-β; Tim-3: T-cell immunoglobulin and mucin-domain containing-3. <sup>1</sup>Licensed by Food and Drug Administration.

CheckMate459 (NCT02576509), is a phase III, randomized, open label trial of nivolumab versus sorafenib which has closed to recruitment and results are awaited at the present time.

There are a number of registered phase III trials looking at PD-1 checkpoint blockade. The ORIENT-32 study (NCT03794440) is a randomized, open-label, multicentre trial in China randomizing patients to a combination of sintilimab (PD-1 inhibitor) and bevacizumab (anti-VEGF antibody) versus a control arm of sorafenib. The RATIONALE-301 study (NCT03412773) is a phase III trial randomizing patients to the PD-1 inhibitor tislelizumab monotherapy versus sorafenib. Pembrolizumab (Keytruda®), another IgG4 isotype antibody targeting the PD-1 receptor of lymphocytes, has been similarly studied as monotherapy. In the 104 patients enrolled in the open-label, phase II trial KEYNOTE-240 (NCT02702401) there were mixed results. An objective response in 17% of patients (complete in 1%, partial in 16%) was offset by serious treatment-related adverse events in 15%, including 1 death associated with ulcerative oesophagitis attributed to treatment<sup>[57]</sup>. However, based on the promising response rates pembrolizumab was granted accelerated approval for HCC. Unfortunately Merck and Co. has recently announced that the subsequent phase III trial of pembrolizumab versus placebo did not meet its co-primary endpoints of overall survival (OS) and progression-free survival in patients with advanced HCC<sup>[58]</sup>. These results, although disappointing, would appear to be consistent with the opinion that checkpoint blockade may well be most efficacious as combination therapy<sup>[54,59]</sup>. Combination of PD-1/PD-L1 blockade may be with VEGF inhibition (NCT03794440, NCT03713593, NCT03764293, NCT03434379), as well as with locoregional treatment or resection (NCT03847428, NCT03755739), or indeed with another checkpoint inhibitor. However, care needs to be taken as combination therapy with checkpoint inhibitors can lead to higher rates of side-effects including an immune-mediated hepatitis<sup>[60]</sup>.

**Cytotoxic T-lymphocyte associated antigen 4:** CTLA-4 is another membrane bound molecule which keeps the immune response in check. It has a multifaceted role, actively competing for binding to the co-stimulatory molecule CD28, and leading to increased secretion of the immunoregulatory cytokine IL-10, as well as serving as a key mediator by which regulatory T cells (Tregs) dampen immune response<sup>[61,62]</sup>. Inhibition of CTLA-4 is associated with improved clinical outcomes in other

Table 1 Phase III trials of checkpoint inhibitors

Trial identifier	Targets	Drugs	Other treatment	Patient group	Status	n	Estimated completion date
NCT03794440	PD-1 VEGF	Sintilimab Bevacizumab biosimilar	vs Sorafenib	Advanced HCC	Recruiting	566	Dec 2022
NCT03298451	CTLA-4 PD-L1	Tremelimumab Durvalumab	vs Sorafenib	HCC BCLC stage B not eligible for locoregional therapy	Recruiting	1310	Jun 2021
NCT02702401	PD-1	Pembrolizumab	vs Placebo	Advanced HCC	Results available	408	Dec 2019
NCT02576509	PD-1	Nivolumab	vs Sorafenib	Advanced HCC	Active, not recruiting	726	July 2020
NCT03755739	PD-1	Pembrolizumab	Peripheral vs hepatic infusion following TACE	Advanced HCC	Recruiting	200	Nov 2021
NCT03062358	PD-1	Pembrolizumab	vs Placebo	Advanced HCC	Recruiting	450	Jan 2022
NCT03713593	PD-1 VEGR	Pembrolizumab Lenvatinib	vs Lenvatinib monotherapy	Advanced HCC	Recruiting	750	July 2022
NCT03847428	PD-L1 VEGF	Durvalumab Bevacizumab	Combination with resection/MWA vs resection/MWA alone	HCC eligible for curative resection/MWA	Not yet recruiting	888	June 2023
NCT03764293	PD-1 TKI	Camrelizumab Apatinib	vs Sorafenib	Advanced HCC	Not yet recruiting	510	Jan 2022
NCT03434379	PD-L1 VEGF	Atezolizumab Bevacizumab	vs Sorafenib	Advanced HCC	Recruiting	480	June 2022

HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; MWA: Microwave ablation; PD-1: Programmed cell death protein 1; TACE: Transcatheter arterial chemoembolization; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

malignancies such as metastatic melanoma. In 2011, ipilimumab (YERVOY®) was the first checkpoint inhibitor approved by the FDA. Reports of therapeutic CTLA-4 blockade in HCC have also shown promise. In 2013 there was a reported phase I trial of 20 patients with advanced HCC and a background of HCV who received the CTLA-4 inhibitor tremelimumab<sup>[63]</sup>. Partial response was 17.6% and disease control was 76.4%. Time to progression was 6.48 mo (95% confidence interval 3.95-9.14). Although intense elevations in transaminases were common, particularly after first dose, no course of steroids were required for hepatotoxicity. The randomized phase III HIMALAYA trial (NCT03298451) is recruiting patients for randomization to a combination of CTLA-4 inhibitor tremelimumab and PD-L1 inhibitor durvalumab versus sorafenib. It leads on from the early clinical data of 40 patients enrolled in the phase I trial of durvalumab and tremelimumab in advanced HCC<sup>[64]</sup>. Patient selection for combination checkpoint blockade will no doubt be key with 20% of patients having at least one grade 3 adverse event.

Furthermore, the combination of checkpoint blockade with locoregional therapy is attractive, with the potential for CTLA-4 inhibition to uncouple the systemic immunogenic response which occurs with tumour necrosis. A phase II trial which enrolled 32 patients (predominantly with HCV) treated with tremelimumab and followed by subtotal RFA or chemoembolization, demonstrated that of the 19 patients with lesions evaluable 5 of them (26%) showed partial response<sup>[65]</sup>.

**T-cell immunoglobulin and mucin-domain containing-3:** TIM-3 is another transmembrane protein which is known to be expressed on CD4<sup>+</sup> T Helper 1 cells and CD8<sup>+</sup> cytotoxic cells<sup>[66]</sup>. Initially identified due to a putative pathogenic association with autoimmune disease, interest in this as a therapeutic has grown due to its role in the ability of tumour cells to evade immunosurveillance. A propensity of CD8<sup>+</sup> cells to co-express both PD-1 and TIM-3 seems to contribute to the dysfunctional phenotype of CD8<sup>+</sup> T cells<sup>[67,68]</sup>. We await with interest a phase II trial of dual blockade of anti-TIM-3 and PD-1 in HCC (NCT03680508) which has not yet begun recruitment.

**Transforming growth factor-β:** TGF-β is a membrane bound molecule expressed on

and associated with a Treg subset which suppresses CD4<sup>+</sup> T cell response in tumour tissue, promoting progression, in both murine models and HCC patients<sup>[69]</sup>. Co-expression of PD-1 on these CD4<sup>+</sup>CD69<sup>+</sup> Tregs makes for another potential combination therapy. Results are awaited of a phase I trial assigning patients in parallel to both the anti-TGF- $\beta$  monoclonal antibody NIS793 and PD-1 inhibitor spartalizumab (NCT02947165) due for completion in April 2021.

**Lymphocyte activation gene 3:** Closely related to CD4, lymphocyte activation gene 3 (LAG-3) is a membrane protein that binds the same ligand, MHC-II<sup>[70]</sup>. Not only do these proteins suppress T cell activity and cytokine release, but they are also of considerable interest due to their upregulation in T cell exhaustion in the context of chronic viral infection or cancer<sup>[71,72]</sup>. The synergistic effect of LAG-3 with PD-1 to induce tumour regression raises another further potential combination therapy<sup>[73]</sup>. Although engineered LAG-3 binding therapy for solid tumours remains in early phase trials, given its significant upregulation in tumour infiltrating CD8<sup>+</sup> T cells of HCC patients, its potential in liver cancer is eagerly awaited<sup>[74]</sup>.

### **Adoptive cell transfer**

In contrast to the active augmentation of immune response seen with checkpoint inhibition therapy, adoptive cell transfer aims to improve HCC outcomes by passively administering autologous lymphocytes following ex vivo cultivation<sup>[75]</sup>. This is a long-standing therapeutic strategy starting over 30 years ago, with infusion of TILs leading to improved responses in metastatic melanoma<sup>[76]</sup>. The broad cell subsets that have been studied in HCC to date include NK cells, cytokine-induced killer (CIK) cells or TILs, and finally chimeric antigen receptor T cells (CAR-T cells).

The first of these, NK cells, form as much as 50% of innate immune cell rich infiltrate within the liver<sup>[26]</sup>. With their ability to kill cells without prior activation or priming they are best known for forming part of the host defence against infection and tumour development<sup>[26]</sup>. In a murine model expanded NK cells exert a significant cytotoxic effect against HCC cells, reducing tumour growth and improving OS. Furthermore, they enhanced the effect of sorafenib in the same study<sup>[77]</sup>. Although clinical data on use is limited, there has been a successfully conducted phase I trial in patients with liver cirrhosis with HCC undergoing liver transplantation. NK cells derived from donor liver perfusate, stimulated with IL-2 and administered showed upregulation of peripheral NK cell cytotoxicity and no adverse events<sup>[78]</sup>. We await ongoing trials of high affinity NK cells versus sorafenib (NCT03563170) and combination therapy of NK cell transfer with irreversible electroporation (IRE) *vs* IRE alone (NCT03008343).

Next are the CIK cells which represent another novel immunotherapeutic option. By incubating peripheral blood monocytes with cytokines including IL-1, IL-2, IFN- $\gamma$  and a monoclonal antibody against the T cell marker CD3, these cells show a significant inhibitory effect on tumorigenesis<sup>[79]</sup>. These MHC-unrestricted cytotoxic lymphocytes are made up of a heterogeneous group of efficient cytotoxic effector cells comprising predominantly CD3<sup>+</sup>CD56<sup>+</sup> T cells, and some CD3<sup>+</sup>CD56<sup>+</sup> NK cells. Trials into reinfusion of CIK cells have predominantly been studied as an adjunctive therapy following surgical resection, with a theoretical base in murine models showing an effect of these cells on micrometastases<sup>[80]</sup>. Early trials randomizing post-curative resection patients to adjuvant CIK cell therapy or no adjuvant showed promising results with a significantly reduced risk of recurrence, but without an improvement in OS<sup>[81,82]</sup>. The largest study to date, involving 230 patients, was a multicenter, randomized, open label phase 3 trial studying CIK cell therapy as adjuvant to RFA, ethanol injection or curative resection. This showed an improvement of 14 mo in recurrence free survival<sup>[83]</sup>. A systematic review and meta-analysis of CIK cell therapy in HCC in Asia reached similar conclusions that in selected patients, progression free survival and recurrence free survival are improved<sup>[84]</sup>.

Antigen specific T cells have also been studied. These include native TILs and also CAR-T cells. A phase I trial studied administration of autologous TILs in 15 patients with HCC post-resection. This showed successful expansion in 88% and there were no serious adverse events (SAEs) reported<sup>[85]</sup>. The incorporation of a chimeric antigen receptor into T cells to modulate their antigen selectivity and signaling offers another exciting prospect for immunotherapy in HCC. Although discovered 30 years ago, CAR-T cell therapy for HCC remained relatively in its infancy until more recently<sup>[86]</sup>. The FDA approved the first two CAR-T cell therapies Kymriah<sup>®</sup> and Yescarta<sup>®</sup> for lymphoma in 2018 and 2017. A plethora of trials into solid tumours have followed in parallel with these breakthroughs in lymphoma.

HCC has a number of tumour associated antigens (TAAs). Selection of an appropriate antigen for CAR-T cells is integral to their success as a prospective immuno-therapeutic option. Given its high expression and association with poor

prognosis in HCC, GPC-3, a member of the glypican family, has been a natural target antigen to study<sup>[87,88]</sup>. There remains one published phase I trial of 13 patients, 8 of whom had lymphodepletion with fludarabine and cyclophosphamide. These were patients with advanced HCC, portal vein invasion or extrahepatic metastases. This has only been published in abstract form to date, but no dose limiting toxicity was identified and there was one SAE of grade 3 fever found<sup>[89]</sup>. We await the published results of a further phase I clinical trial (NCT02723942) that was completed in 2017. There are currently five Phase I/II trials recruiting, four of which examining GPC-3 and one EpCAM (NCT03198546, NCT03130712, NCT02715362, NCT03013712, NCT02723942). AFP is another potential target TAA that is being explored<sup>[90,91]</sup>. Unfortunately the propensity of AFP to be found on healthy hepatocytes has stymied its potential as a target antigen of CAR-T cell or other targeted immunotherapies.

### Vaccines

Tumour vaccines are agents which increase specific immune responses to tumour antigens. Registered clinical trials for such tumour vaccines in HCC are currently relatively few compared to those studying adoptive cellular therapies and checkpoint inhibitors, in part because of previously disappointing trial results, and also relative lack of efficacy of other tumour vaccines. This may be related to the previous difficulty in identifying the correct tumour antigens, which has now become possible through recent technological breakthroughs allowing massive parallel DNA sequencing. Thus, priming an immune response whether in isolation, or more likely in combination with an immune modulator remains an attractive therapeutic strategy for HCC. A number of agents have been examined to date with regards to this.

**Dendritic cells:** DCs are professional antigen presenting cells, responsible for a multitude of tasks, including absorption, processing and presentation of TAAs. Allogeneic DCs form one broad subset of vaccines by providing both the antigen and the secondary co-stimulation required to prime an effective T cell response. Isolating DCs from peripheral blood, expanding them *ex vivo* and stimulating with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) produces primed DCs for reinfusion. The injection of these cells to induce recruitment of effector cells and provoke a cascade of tumour lysis and further TAA release, is another attractive, targeted mechanism<sup>[92]</sup>. A number of techniques may be employed to optimize this TAA priming and enhance the efficacy of the vaccine. DCs can be transduced with DNA or RNA encoding known TAAs, or they may be incubated with tumour lysate or fusion of DCs and tumour cells<sup>[93]</sup>.

A recently published phase I trial studied intra-tumoral injection of ilixadencel (pro-inflammatory allogeneic DCs stimulated by GM-CSF and IL-4) either as monotherapy or in combination with sorafenib in 17 patients. The primary objective was to evaluate tolerability. Only one grade 3 adverse event was recorded. 73% of the 15 evaluable patients demonstrated increased tumour specific CD8<sup>+</sup> T cells in peripheral blood, suggesting a successful immune provoked response at least<sup>[94]</sup>.

**Peptide vaccines:** Peptide vaccines constitute an alternative option in terms of generating an effective immune reaction. However, although there has been success in terms of immunological surrogates such as generating GPC-3 reactive cytotoxic T lymphocytes in one phase I trial, this has not translated into clinical successes<sup>[95]</sup>. Despite a plethora of TAAs identified in HCC only trials utilizing AFP, GPC-3 and MRP3 have shown any success inducing a T cell response rate over 70%, with other TAAs such as SSX-2, NY-ESO-1, hTERT and MAGE-A all inducing much lower rates<sup>[96]</sup>.

**Oncolytic viruses:** A more recent development in the arena of tumour vaccines is the use of oncolytic viruses. These therapeutically useful viruses are targeted to preferentially replicate in cancer cells. To date they have been predominantly introduced by intra-tumoral injection. The modified poxvirus JX-594 remains the lead oncolytic virus of interest in clinical trials with regards to HCC. As an immunotherapeutic agent it piqued considerable interest when it conferred a dose-related survival benefit (median of 14.1 mo compared to 6.7 mo) in a phase II dose-finding trial of 30 patients<sup>[97]</sup>. The global, randomized, open-label, phase III study of Pexa-Vec (JX-594; an oncolytic vaccinia virus which selectively targets cancer cells) is currently recruiting patients with advanced HCC to two arms of vaccination with sorafenib vs. sorafenib alone<sup>[98]</sup>. We eagerly await the results of this particularly as a combination therapy.

## CONCLUSION



Immunotherapy for HCC is still in its infancy compared to other tumours. Encouraging results with PD-1 inhibitors are emerging, and prospects for combination therapies arising. This makes immunological sense, as the immune system is a multi-faceted and integrated effector system. Optimising this response is challenging, especially because of the immune environment on which HCC arises, and the challenges of treating an individual with cirrhosis, which substantially decreases the therapeutic index of these agents. Nevertheless, the massive interest in immunotherapy, gives hope that better combinations of drugs will be found to treat this challenging disease.

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## Application of Big Data analysis in gastrointestinal research

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### Abstract

Big Data, which are characterized by certain unique traits like volume, velocity and value, have revolutionized the research of multiple fields including medicine. Big Data in health care are defined as large datasets that are collected routinely or automatically, and stored electronically. With the rapidly expanding volume of health data collection, it is envisioned that the Big Data approach can improve not only individual health, but also the performance of health care systems. The application of Big Data analysis in the field of gastroenterology and hepatology research has also opened new research approaches. While it retains most of the advantages and avoids some of the disadvantages of traditional observational studies (case-control and prospective cohort studies), it allows for phenomapping of disease heterogeneity, enhancement of drug safety, as well as development of precision medicine, prediction models and personalized treatment. Unlike randomized controlled trials, it reflects the real-world situation and studies patients who are often under-represented in randomized controlled trials. However, residual and/or unmeasured confounding remains a major concern, which requires meticulous study design and various statistical adjustment methods. Other potential drawbacks include data validity, missing data, incomplete data capture due to the unavailability of diagnosis codes for certain clinical situations, and individual privacy. With continuous technological advances, some of the current limitations with Big Data may be further minimized. This review will illustrate the use of Big Data research on gastrointestinal and liver diseases using recently published examples.

**Key words:** Healthcare dataset; Epidemiology; Gastric cancer; Inflammatory bowel disease; Colorectal cancer; Hepatocellular carcinoma; Gastrointestinal bleeding

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**Core tip:** Digital collection and storage of data has led to the generation of Big Data. Big Data analysis in the field of gastroenterology and hepatology allows for phenomapping due to disease heterogeneity (*e.g.*, inflammatory bowel disease, gastrointestinal and liver cancers) and hence the development of precision medicine, enhances in drug safety and faster drug discovery. It has also revolutionized clinical study approaches. Although there are still limitations to Big Data approaches, some of them may be further minimized with continuous technological advances.

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## INTRODUCTION

The etymology of “Big Data” can be dated back to the 1990s, and this term has become popular after John Mashey, the then chief scientist at Silicon Graphics<sup>[1]</sup>. Datasets are exponentially expanding every day, fed with a wide array of sources<sup>[2]</sup> like mobile communications, websites, social media/crowdsourcing, sensors, cameras/lasers, transaction process-generated data (*e.g.*, sales queries, purchases), administrative, scientific experiments, science computing, and industrial manufacturing. The application of Big Data analysis has proven successful in many fields. Technology giants (*e.g.*, Amazon, Apple, Google) have boosted sales and increased revenue by means of Big Data approaches<sup>[3]</sup>. It has also been adopted as part of the electoral strategies in political campaigns<sup>[4]</sup>.

There is currently no consensus on the definition of Big Data, but the characteristics pertinent to the process of collection, storage, processing and analysis of these data helps to forge Big Data as a more tangible term. It was first described by Doug Laney in 2001 that Big Data possessed 3Vs: Volume (storage space necessary for data recording and storage), Velocity (speed of data generation and transformation) and Variety (various data sources)<sup>[5]</sup>. Since then, many other traits to define Big Data have been proposed, including veracity, value, exhaustivity ( $n = \text{all}$ ), fine-grained resolution, indexicality, relationality, extensionality, scalability, and variability<sup>[2]</sup>.

## BIG DATA RESEARCH IN GASTROENTEROLOGY AND HEPATOLOGY

The digitalization of nearly every aspect of daily life has made no exception in the field of healthcare, with the importance of Big Data application being increasingly recognised and advocated in recent years. While there are various definitions of Big Data outside of the medical field, the specific definition with respect to health has only been proposed in recent years. According to the report produced under the third Health Programme (2014-2020) from the Consumer, Health, Agriculture and Food Executive Agency mandated by the European Commission<sup>[6]</sup>, Big Data in Health are defined as large datasets that are collected routinely or automatically, and stored electronically. It merges existing databases and is reusable (*i.e.*, multipurpose data that are not intended for a specific study), with the aim of improving health and health system performance. A further supplement is the scale and complexity of the data that mandates dedicated analytical and statistical approaches<sup>[7]</sup>. Such large volume and scale of Big Data arise not only from the number of subjects included, but also the diversity of variables from different domains (clinical, lifestyle, socioeconomic, environmental, biological and omics) at several time points. The estimated healthcare volume of 153 exabytes ( $10^{18}$ ) in 2014 is projected to hit 2,300 exabytes by 2020<sup>[8,9]</sup>.

Big Data in Health relies on a wealth of sources: Administrative databases, insurance claims, electronic health records, cohort study data, clinical trial data, pharmaceutical data, medical images, biometric data, biomarker data, omics data (*e.g.*, genomics, proteomics, metabolomics, microbiomics), social media (*e.g.*, Facebook, Twitter), income statistics, environmental databases, mobile applications, e-Health tools, and telemedicine (diagnosis and management at a distance, particularly by means of the internet, mobile phone applications and wearable devices)<sup>[9]</sup>. The

importance of “data fusion” therefore relies on the systematic linking of datasets from different sources to add values and new insights, enabling the analysis of health data from different perspectives (individual, group, social, economic and environmental factors) across different regions or nations.

Disease entities in the field of gastroenterology and hepatology are often heterogeneous [*e.g.*, malignancy, inflammatory bowel disease (IBD)] with a wide range of clinical phenotypes (*e.g.*, age of onset, severity, natural course of disease, association with other diseases, treatment response). Big Data analysis allows for the subclassification of a disease entity into distinct subgroups (*i.e.*, phenomapping), which enhances understanding of disease pathogenesis, as well as the development of more precise predictive models of disease outcomes. The use of only clinical and laboratory data (as in traditional clinical research) in predicting disease course, outcome and treatment response may not achieve a high accuracy<sup>[9]</sup>. Similarly, although genome-wide association studies (commonly known as GWAS) and identification of single nucleotide variants have linked particular disease phenotypes to genetic defects, most genetic variants have a small impact on disease risk, behaviour and treatment response<sup>[10]</sup>. This inaccurate differentiation has led to the unnecessary use of therapeutics (which are sometimes costly with undesirable side effects) in many patients (*e.g.*, biologics in IBD patients). It therefore appears that only by considering the complex interactions between genetic, lifestyle, environmental factors, and previously unconsidered factors (*e.g.*, omics) in Big Data approaches can a reliable predictive prognostic model be developed, which ultimately guides a targeted approach for selecting treatment regimens for individual patients (*i.e.*, precision or personalized medicine)<sup>[9,11,12]</sup>.

Apart from phenomapping and precision medicine, other important implications of Big Data approaches are drug discovery and safety. Drug research and development (R and D) is an expensive and lengthy process, with each drug approval costing \$3.2–32.3 billion US dollars<sup>[13]</sup>. Many of the trial drugs have proven futile or harmful in early or even late stages of the development (*e.g.*, secukinumab in Crohn’s disease<sup>[14]</sup>). Even for drugs proven to be beneficial, they may only work in certain subgroups of patients. The heterogeneity of therapeutic outcomes is again likely multifactorial. Precision medicine from Big Data approaches will help pharmaceutical companies predict drug action and prioritize drug targets on a specific group of patients<sup>[15]</sup>. This ensures a cost-effective approach in developing new therapeutics with a lower chance of futility.

Recently, “drug repositioning” or “drug repurposing” has been advocated, in which currently approved drugs are explored for other indications of gastrointestinal and hepatic diseases. However, to make sense of the large-scale genomic and phenotypic data, advanced data processing and analysis is an indispensable element, hence giving rise to the term “computational drug repositioning or repurposing”<sup>[16]</sup>. This involves a process of various computational repositioning strategies utilizing different available data sources, computational repositioning approaches (*e.g.*, machine learning, network analysis, text mining and semantic inference), followed by validation *via* both computational (electronic health records) and experimental methods (*in vitro* and *in vivo* models). Applicable disease areas include oncology [*e.g.*, hepatocellular carcinoma (HCC)]<sup>[17,18]</sup>, infectious diseases, and personalized medicine, just to name a few. New indications of existing medications constituted 20% of 84 drugs products introduced to the market in 2013<sup>[19]</sup>. Drug repositioning is expected to play an increasingly important role in drug discovery for gastrointestinal and liver diseases.

With regards to drug safety, monitoring currently relies on data from randomized controlled trials (RCTs) or post-marketing studies. However, RCTs may be underpowered to detect rare but important side effects, and fail to capture adverse effects that only manifest beyond the designed follow-up time (*e.g.*, malignancy). Post-marketing studies based on registries are resource-intensive in terms of cost and time, and the safety profile of a drug can only be depicted several years after marketing. The application of text mining, the computational process of extracting meaningful information from unstructured text, has proven useful to improve pharmacovigilance (*e.g.*, arthralgia in vedolizumab users in IBD<sup>[20]</sup>). The sources are not limited to medical literature and clinical notes, but also product labelling, social media and web search logs<sup>[21,22]</sup>.

## ADVANTAGES AND SHORTCOMINGS OF BIG DATA APPROACHES

In healthcare research, RCT is regarded as the gold standard to investigate the

causality between exposure and the outcome of interest. Randomization balances prognostic factors across intervention and control groups. It eliminates both measured and unmeasured confounding, making the establishment of causality possible. However, it is resource-intensive to conduct RCTs in terms of money, manpower and time. It is difficult to study rare events (*e.g.*, cancer, death) or long-term effects. Due to the stringent inclusion and exclusion criteria, as well as differential levels of care and follow-up in a clinical trial setting, results from RCTs may not reflect real-life situations, and may not be generalizable to other populations. Finally, effects of harmful exposure cannot be studied due to ethical concerns.

To circumvent these shortcomings of RCTs, observational studies are alternatives. Case-control studies are cheaper and quicker to conduct, and can study multiple risk factors of rare diseases, as well as potentially harmful exposure that is otherwise impossible in RCTs. On the other hand, prospective cohort studies can investigate multiple exposures and outcomes, effects of rare exposure, as well as potentially harmful exposure. Nonetheless, it is difficult to study rare exposures in case-control studies, as well as rare diseases or long-term effects in prospective cohort studies. It is also impossible and unethical to prospectively follow the natural history of chronic diseases and its complications without appropriate interventions<sup>[23]</sup>. In addition, for both study designs, multiple biases (*e.g.*, reverse causality, selection bias, interviewer bias, recall bias) can exist, and confounding, whether measured or unmeasured, is always possible.

The application of Big Data analysis in healthcare research has revolutionized clinical study approaches. Clinical studies making use of these datasets usually belong to either retrospective cohort studies (non-concurrent/historical cohort studies) or nested case-control studies. As the clinical data are readily available without delays, and easily retrieved from the electronic storage system, a multitude of risk factors can be included to analyse the outcome. It also enables the study of rare exposures, rare events and long-term effects within a relatively short period of time. Resources are much less than that required for prospective cohort study design, except for dedicated manpower with the aid of high-performance computers and software, *e.g.*, R, Software for Statistics and Data Science, Statistics Analysis System, Python. In essence, it retains most of the advantages while avoiding some of the disadvantages of case-control and prospective cohort studies. Unlike RCTs, it reflects the real-world efficacy, and studies patients who are often under-represented in or completely excluded from RCTs (*e.g.*, the elderly, pregnant women). Furthermore, the huge sample size of Big Data permits subgroup analysis to investigate interactions between different variables with the outcome of interest without sacrificing statistical power. It enables the investigation of varying effects due to time factors (*i.e.*, division of the follow-up duration into different segments) on the association between exposure and outcome, given a sufficiently long observation period (in terms of years or decades) and sample size. It also allows for multiple sensitivity analyses by including certain sub-cohorts, modifying definitions of exposure (*e.g.*, duration of drug use), or different statistical methods to prove the robustness of study results. A reliable capture of small variations in incidence or flares of a disease according to temporal variations also heavily depend on the sample size. In the most ideal situation of  $n = \text{all}$ , selection bias will no longer be a concern.

However, it should be acknowledged that without randomization, residual and/or unmeasured confounding remains a concern in Big Data research. As such, one may argue that causality cannot be established. The inclusion of RCT datasets with the extensive collection of data and outcomes for trial participants or linkage with other data sources may partly address this issue<sup>[24]</sup>. The possibility of causality can also be strengthened *via* the fulfilment of the Bradford Hill criteria<sup>[25]</sup>. Second, data validity concerning the accuracy of diagnosis codes (*e.g.*, International Classification of Diseases) in electronic databases has been challenged<sup>[26]</sup>. In addition, milder disease tends to be omitted in the presence of more serious disease, and hence the absence of a diagnosis code may not signify the absence of that particular disease<sup>[27]</sup>. For instance, depression, which is often not coded among the elderly with other serious medical diseases, may be paradoxically associated with reduced mortality. To a certain extent, data validity can be verified through validating the diagnosis codes by cross referencing the actual diagnosis of a subset of patients in the medical records.

Third, missing data can potentially bias the result *via* a differential misclassification bias. There are different remedies, although the use of multiple imputation is preferred, which involves constructing a certain number of complete datasets (*e.g.*,  $n = 50$ ) by imputing the missing variables based on the logistic regression model<sup>[28]</sup>. Nonetheless, missing data with differential misclassifications are not a major problem in Big Data health research, as diagnosis codes are recorded by healthcare professionals, with other clinical/laboratory information being automatically recording in electronic systems. This is unlike questionnaire studies in which missing

data occur due to patient preferences to reveal their details (*i.e.*, misclassification bias).

Fourth, some clinical information may be too sophisticated to be recorded<sup>[26]</sup> (*e.g.*, lifestyle factors, dietary pattern, exercises), incompletely or selectively recorded (*e.g.*, smoking, alcohol use, body mass index, family history), or not represented by the coding system (*e.g.*, bowel preparation in colonoscopy research). This may be partially addressed by using other variables as proxies for unmeasured variables. For example, chronic pulmonary obstructive disease is a surrogate marker of heavy smoking. Certainly, in the most ideal situation, adjusting for a perfect proxy of an unmeasured variable achieves the same effect as adjusting for the variable itself. Large healthcare datasets will usually contain a sufficient set of measured surrogate variables, insofar as it represents an overall proxy for relevant unmeasured confounding. A more fascinating and precise approach is the analysis of unstructured data within the electronic health records [*e.g.*, natural language processing (NLP) to extract meaningful data from text-based documents that do not fit into relational tables]<sup>[29]</sup>. As an example, free-text searches outperformed discharge diagnosis coding in the detection of postoperative complications<sup>[30]</sup>. In the field of pharmacoepidemiological studies, over-the-counter medication usage is frequently not captured in electronic database systems. These “messy data” (false, imprecise or missing information), more often representing non-differential misclassification bias instead of a differential one, will usually attenuate any positive association, and even trend towards null<sup>[23]</sup>. Generally, a “false-negative” result is preferred to a “false-positive” one in epidemiological studies.

Lastly, ethical concerns over an individual’s right to privacy *versus* the common good have yet to be satisfactorily addressed<sup>[31]</sup>. The issue of privacy can be tackled with de-identification of individuals using anonymous identifiers (*e.g.*, unique reference keys in terms of numbers and/or letters), although in rare occasions a remote possibility of discerning individuals still exists<sup>[23]</sup>. For instance, individuals with a very rare disease may be identified *via* mapping with enough geographical detail.

Although Big Data analysis generates hypothesis-free predictive models wherein no clear explanation accountable for the outcome may be found, it provides a valuable opportunity to derive hypotheses based on these observations, which may not be otherwise conceivable. This strategy (in silico discovery and validation) applies to both candidate biomarkers and therapeutic targets to accelerate the development process for an earlier clinical application. In the end, traditionally hypothesis-driven scientific method research should still be applied to validate the results in multi-centre, prospective studies or RCTs. **Table 1** summarizes the advantages and shortcomings of Big Data analysis in gastroenterology and hepatology research, as well as its proposed solutions.

## PROPSENSITY SCORE METHODOLOGY IN BIG DATA ANALYSIS

As stated previously, confounding is an inevitable problem of observational studies, irrespective of the sample size. Confounding is a systematic difference between the group with the exposure of interest and the control group<sup>[27]</sup>. It arises when other factors that affect the exposure of interest are also independent determinants of the outcome. Common sources of confounding include confounding by indication/disease severity, confounding by functional status and cognitive impairment, healthy user/adherer bias, ascertainment bias, surveillance bias, access to healthcare, selective prescription, and the treatment of frail and very sick patients<sup>[27]</sup>.

Propensity score (PS) methodology has become a widely accepted and popular approach in Big Data analysis of analytic studies in healthcare research. A PS is the propensity (probability) of an individual being assigned to an intervention/exposure conditional on other given covariates, but not the outcome<sup>[32]</sup>. It is derived from the logistic regression model by regressing the covariates (exclusive of the outcome) onto the exposure of interest. By taking into account this single score in further statistical analysis, a balance of the characteristics between exposure and control groups could theoretically be achieved in the absence of unmeasured confounding. PS methodology entails PS matching, PS stratification/subclassification, PS analysis by inverse probability of treatment weighting, PS regression adjustment, or a combination of these methods, and we refer readers to other articles for further details<sup>[33]</sup>.

To control for confounding, outcome regression models are traditionally applied. However, this is constrained by the dimensionality of available variables in healthcare datasets (*i.e.*, “curse of dimensionality”). In the simulation study on logistic regression analysis by Peduzzi *et al.*<sup>[34]</sup>, a low events per variable (EPV) was found to be more

**Table 1 Advantages and shortcomings of Big Data analysis (with proposed solutions)**

<b>Advantages</b>	
Clinical data readily available with minimal resources required	
Can study rare exposures	
Can study rare events	
Can study long-term effects	
Real-world data	
Large sample size	
Subgroup analysis	
Sensitivity analysis	
Interaction of different variables	
Adjustment of outcome to a multitude of risk factors	
Precise estimation of effect size	
Reliable capture of small variations in incidence or disease flare	
No selection bias if $n = \text{all}$	
<b>Shortcomings specific of Big Data analysis</b>	<b>Solution</b>
Data validity	Cross reference with medical records in a subset of the sample
Missing data	Statistical methods to deal with missing data, <i>e.g.</i> multiple imputation
	Text mining or natural language processing of unstructured data
Incomplete capture of variables or unavailability of certain diagnosis codes	Surrogate markers ( <i>e.g.</i> , COPD for smoking, alcohol-related diseases for alcoholism)
	Inclusion of a large set of measured variables
	Text mining or natural language processing of unstructured data
Privacy	De-identification of individuals
	Review of study plan by local ethics committee
Hypothesis-free predictive models	Validation in prospective studies or randomized control trials
<b>Shortcomings of all observational study including Big Data analysis</b>	<b>Solution</b>
Residual and/or unmeasured confounding	Inclusion of a large set of measured variables
	Inclusion of RCT datasets with extensive collection of data and outcomes for trial participants or linkage with other data sources
	Fulfilment of Bradford Hill criteria
Reverse causality/protopathic bias (outcome of interest leads to exposure of interest)	Cohort study design instead of case-control study design
Example: Early symptoms of undiagnosed GC leads to PPI use, rather than PPIs cause GC	Excluding prescriptions of drugs of interest ( <i>e.g.</i> , PPIs) within a certain period ( <i>e.g.</i> , 6 mo) before development of the outcome of interest ( <i>e.g.</i> , gastric cancer)
Selection bias	Encompassing entire study population ( $n = \text{all}$ )
Indication bias (or confounding by indication/disease severity)	Balance of patient characteristics, in particular comorbidities that are indications for a certain treatment ( <i>e.g.</i> , PS matching of a large set of measured variables)
	Negative control exposure
Confounding by functional status and cognitive impairment	Balance of patient characteristics, in particular comorbidities that can affect functional and cognitive status ( <i>e.g.</i> , PS matching)
Healthy user bias / adherer bias (individuals who are more health conscious tend to have better health outcomes)	Adjustment for other lifestyle factors – text mining or natural language processing of unstructured data
Immortal time bias (arises when the study outcome cannot occur during a period of follow-up due to study design)	Landmark analysis
	Analysis using time varying covariates
Ascertainment bias / surveillance bias / detection bias (differential degree of surveillance or screening for the outcome among exposed and unexposed individuals) Example: PPI users may undergo upper endoscopy more frequently than non-PPI users, and hence more GC detected in PPI users	Selection of an unexposed group with a similar likelihood of screening/ testing
	Selection of an outcome that are likely to be diagnosed equally in exposed and control groups
	Adjustment for the surveillance rate
Access to healthcare	Stratified analysis according to patients' residential regions ( <i>e.g.</i> , rural <i>vs</i> urban), socioeconomic status, immigration status, race/ethnicity, institutional factors ( <i>e.g.</i> , restrictive formularies)
Selective prescription and treatment in frail and very sick patients	PS methodology (trimming of areas of non-overlap, PS matching, PS by treatment interaction)

COPD: Chronic pulmonary obstructive disease; RCT: Randomized controlled trial; GC: Gastric cancer; PPI: Proton pump inhibitor; PS: Propensity score.



influential than other problems, such as sample size or the total number of events. If the number of EPV is less than ten, the regression coefficients may be biased in both positive and negative directions, the sample variance of the regression coefficients may be over- or under-estimated, the 95% confidence interval may not have proper coverage, and the chance of paradoxical associations (significance in the wrong direction) may be increased. The use of PS methodology, by condensing all covariates into one single variable (PS), can thus address this “curse of dimensionality”<sup>[35]</sup>. However, PS methodology may not offer additional benefits if the EPV is large enough. Statistical significance differs between the two methods in only 10% of cases, in which traditional regression models give a statistically significant association not otherwise found in PS methodology<sup>[36]</sup>. In addition, the effect estimate derived by traditional models differs by more than 20% from that obtained by PS methodology in 13% of cases<sup>[37]</sup>.

The use of PS allows the recognition of subjects with absolute indications (or contraindications) of an intervention, who have no comparable unexposed (or exposed) counterparts for valid estimation of relative or absolute differences in the outcomes<sup>[35]</sup>. This can be easily identified by plotting a graph of PS distribution between the two groups to look for areas of non-overlap. This pitfall is unlikely to be recognised by traditional modelling, and could be influential as a result of effect measure modification or model misspecification. PS methodology allows trimming (*i.e.*, excluding individuals with areas of non-overlap in PS distributions) or matching to ensure comparability between exposure and control groups. In particular, PS matching does not make strong assumptions of linearity in the relationship of propensity with outcome, and is also better than other matching strategies to achieve an optimal balance of a large set of covariates. The interaction effect of PS with treatment may exist, as effectiveness of an intervention varies according to the indications. An intervention is beneficial in patients with clear indications, but paradoxically provides no benefit, or is even harmful in those with weak indications or contraindications. This was nicely illustrated in the study by Kurth *et al*<sup>[38]</sup> on the effect of tissue plasminogen activator on in-hospital mortality. Table 2 summarizes the major advantages of PS methodologies.

## EXAMPLES OF GASTROINTESTINAL DISEASE RESEARCH USING BIG DATA APPROACHES

Tables 3-7 show a list of research using Big Data approaches from different regions/countries worldwide. This list is by no means exhaustive, however provides a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. Specifically, in the following section, we will demonstrate how researchers conducted research on some important gastrointestinal and liver diseases, including gastric cancer, gastrointestinal bleeding (GIB), IBD, colorectal cancer (CRC), and HCC. It should be noted that the majority of database systems fulfil the characteristics of the 3Vs (volume, velocity and variety). This is with the exception of the Nurses Health Study (known as NHSII) and Health Professionals Follow-up Study (known as HPFS), which are prospective studies without instantaneous updates of the clinical information using participant questionnaires, thus limiting the velocity of data generation and transformation.

### Gastric cancer

Gastric cancer is the fifth most common cancer and third leading cause of cancer-related deaths worldwide<sup>[39]</sup>. Around two-thirds of patients have gastric cancer diagnosed at an advanced stage, rendering curative surgery impossible<sup>[40,41]</sup>. Infection by *Helicobacter pylori* (*H. pylori*), a class I human carcinogen<sup>[42]</sup>, confers a two- to three-fold increase in gastric cancer risk<sup>[43,44]</sup>. RCTs and prospective cohort studies on the effect of *H. pylori* eradication on gastric cancer development are difficult to perform due to the low incidence of gastric cancer, as well as the long lag time of any potential benefits, which mandate a huge sample size with long follow-up duration.

However, Big Data analysis may shed new light on the role of *H. pylori* eradication on gastric cancer development based on population-based health databases. It was shown in a Swedish population-based study that *H. pylori* eradication therapy was associated with a lower gastric cancer risk compared with the general population, but this effect only started to appear beyond 5 years post-treatment<sup>[45]</sup>. Stratified analysis in a Taiwanese study based on the National Health Insurance Database (commonly known as NHID) showed that early *H. pylori* eradication was associated with a lower gastric cancer risk than late eradication when compared with the general population<sup>[46]</sup>. Based on a territory-wide public healthcare database in Hong Kong

Table 2 Advantages of propensity score methodology

Advantages	Remarks
Addressing “curse of dimensionality” when EPV < 10	Traditional multivariable regression models yield similar results if EPV ≥ 10
Recognition of subjects with absolute indications (or contraindications) of an intervention	Exclusion of areas of non-overlap of the PS distribution between exposed and unexposed groups to ensure comparability
Identification of PS interaction with treatment	Variation of effectiveness of an intervention according to indications (PS) may only be identified <i>via</i> stratified analysis by PS

EPV: Events per variable; PS: Propensity score.

called the Clinical Data Analysis and Reporting System, *H. pylori* eradication therapy was beneficial even in older age groups ( $\geq 60$  years)<sup>[47]</sup>. Apart from *H. pylori* eradication, regular non-steroidal anti-inflammatory drug use was also shown to be a protective factor for gastric cancer based on the study from NHID from Taiwan<sup>[48]</sup>. Long-term aspirin use further reduced gastric cancer risk in patients who had received *H. pylori* eradication therapy<sup>[49]</sup>. Moreover, the long-term use of metformin was associated with a lower gastric cancer risk in our patients who had received *H. pylori* eradication therapy<sup>[50]</sup>.

On the other hand, long-term proton pump inhibitor (PPI) use was associated with an increased gastric cancer risk in patients who had received *H. pylori* eradication therapy<sup>[51]</sup>, which is otherwise difficult to be addressed by RCTs<sup>[52]</sup>. This finding was echoed by another nationwide study<sup>[53]</sup>. A study on the interaction between aspirin and PPIs further showed that PPIs were associated with a higher cancer risk among non-aspirin users, but not among aspirin users<sup>[54]</sup>. However, pantoprazole, a long-acting PPI, was not associated with an increased gastric cancer risk compared with other shorter-acting PPIs in a United States Food and Drug Administration (commonly known as FDA)-mandated study<sup>[55]</sup>. Other risk factors for gastric cancer determined by large healthcare datasets included the extent of gastric intestinal metaplasia, as well as a family history of gastric cancer<sup>[56]</sup>. In addition, racial/ethnic minorities had a 40%-50% increase in gastric cancer risk compared with the Hispanic and white populations<sup>[57]</sup>.

### GIB

Upper GIB is one of the most common causes of hospitalization, and emergency department visits that pose significant economic burdens on the healthcare system. Antiplatelet agents (including aspirin and P2Y<sub>12</sub> inhibitors) were major causative agents<sup>[5]</sup>. In a nationwide retrospective cohort study, it was shown that *H. pylori* eradication and PPIs were associated with reduced incidences of gastric ulcer (42%-48%) and duodenal ulcers (41%-71%)<sup>[58]</sup>. However, importantly, concomitant use of clopidogrel, H<sub>2</sub>-receptor antagonists (referred to as H<sub>2</sub>RAs) and PPIs was associated with an increased risk of acute coronary syndrome or all-cause mortality<sup>[59]</sup>. This harmful effect was particularly prominent for PPIs with high CYP2C19 inhibitory potential<sup>[60]</sup>. These findings raised the need for judicious use of gastroprotective agents in clopidogrel users, and called for further studies to determine causality *versus* biases (*e.g.*, indication bias).

When novel oral anticoagulants (NOACs) were first introduced, there was a paucity of real-world data on the GIB risk and its preventive measures<sup>[61]</sup>. In a territory-wide retrospective cohort study, the risk of GIB was determined in dabigatran users, with risk factors identified and effects of gastroprotective agents (PPIs and H<sub>2</sub>RAs) investigated<sup>[62]</sup>. All patients who were newly prescribed dabigatran were identified ( $n = 5041$ ). There were 124 (2.5%) GIB cases, with an incidence rate of GIB of 41.7 cases per 1,000 person-years. PPIs were found to protect against upper GIB. This important finding has recently been echoed by an even larger-scale study involving more than 3 million NOAC users<sup>[63]</sup>, with a consistent beneficial effect of PPIs on upper GIB across various NOACs (dabigatran, rivaroxaban and apixaban). Head-to-head comparisons between different NOACs and their interaction with PPIs would barely be possible in other study designs, given the huge number of study subjects required to ensure statistical power. These drug safety data can be easily ascertained by Big Data analysis of electronic health databases, which would be otherwise difficult in other observational studies or RCTs due to the various limitations previously mentioned, especially if the absolute risk difference is small.

### IBD

Precise outcome prediction in IBD remains challenging, as it is a highly heterogeneous

Table 3 Examples of studies on gastric cancer research by utilization of large healthcare datasets

Gastric cancer					
Country/Region	Database	Area of research	Sample size	Design, statistical methods and 3V	Application
Taiwan, China	Taiwan National Health Insurance Database (NHID)	GC Wu <i>et al</i> <sup>[46]</sup> , 2009	80255	Nationwide retrospective cohort study  Comparison with general population to derive SIR  Volume, Velocity and Variety	Early <i>vs</i> late <i>H. pylori</i> eradication on GC risk
		GC Wu <i>et al</i> <sup>[48]</sup> , 2010	52161	Nationwide retrospective cohort study  Comparison with general population to derive SIR  Volume, Velocity and Variety	Association between NSAIDs and GC
Hong Kong, China	Clinical Data Analysis and Reporting System (CDARS)	GC Cheung <i>et al</i> <sup>[51]</sup> , 2018	63397	Territory-wide retrospective cohort study  PS regression adjustment  Volume, Velocity and Variety	Association between PPIs and GC
		GC Cheung <i>et al</i> <sup>[49]</sup> , 2018	63605	Territory-wide retrospective cohort study  PS regression adjustment  Volume, Velocity and Variety	Association between aspirin and GC
		GC Leung <i>et al</i> <sup>[47]</sup> , 2018	63397	Territory-wide retrospective cohort study  Comparison with general population to derive SIR  Volume, Velocity and Variety	Effect of <i>H. pylori</i> eradication among different age groups
		GC Cheung <i>et al</i> <sup>[50]</sup> , 2018	7266	Territory-wide retrospective cohort study  PS regression adjustment  Sensitivity analysis: PS weighting by IPTW and PS matching  Volume, Velocity and Variety	Association between metformin and GC
Sweden	Swedish Cancer Registry	GC Brusselaers <i>et al</i> <sup>[53]</sup> , 2017	797067	Nationwide retrospective cohort study  Comparison with general population to derive SIR  Volume, Velocity and Variety	Association between PPIs and GC
	Swedish Prescribed Drug Registry	GC Doorakkers <i>et al</i> <sup>[45]</sup> , 2018	95176	Nationwide retrospective cohort study  Comparison with general population to derive SIR	Effect of <i>H. pylori</i> eradication on GC risk

United States	Kaiser Permanente (KP)	GC	61684	Volume, Velocity and Variety	Association between different PPIs and GC
				Retrospective cohort study	
				Volume, Velocity and Variety	

This list is not exhaustive, but serves to provide a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. 3V: Volume/velocity/variety; GC: Gastric cancer; SIR: Standardized incidence ratio; *H. pylori*: *Helicobacter pylori*; NSAIDs: Non-steroidal anti-inflammatory drugs; PS: Propensity score; PPIs: Proton pump inhibitors; IPTW: Inverse probability of treatment weighting.

disease with numerous predictive factors. Machine learning algorithms are particularly useful in deriving predictive models, including risk factors<sup>[64]</sup>, disease outcomes<sup>[65]</sup> and treatment responses<sup>[66,67]</sup>, hence allowing the identification of at-risk individuals who require early aggressive intervention. Today, there is still an unmet need for newer therapeutic agents for IBD, as the long-term efficacy of current options including anti-tumour necrosis factor (anti-TNF) and anti-integrin  $\alpha_4\beta_7$  are still unsatisfactory. However, the process of new drug discovery for IBD is prolonged and costly, and success is not guaranteed. For instance, mongersen, an antisense oligonucleotide showing a promising effect in a phase II trial in Crohn's disease<sup>[68]</sup>, was prematurely terminated in the phase III program<sup>[69]</sup>. The results for secukinumab, an anti-IL-17A monoclonal antibody, was also disappointing in moderate to severe Crohn's disease, in which it was less effective and carried higher rates of adverse events compared with placebo<sup>[14]</sup>, despite the potential role of IL-17 in Crohn's disease as suggested by animal models and GWAS. Drug repurposing from Big Data applications helps in this regard, as illustrated by Dudley *et al*<sup>[70]</sup>. In that study, computational approaches were used to discover new drugs for IBD *in silico* by comparing the gene expression profiles from 164 drug compounds to a gene expression signature of IBD from publicly available data obtained from the NCBI Gene Expression Omnibus<sup>[70]</sup>. A technique, called "signature inversion"<sup>[16]</sup>, was used to identify drugs that can reverse a disease signature (transcriptomic, proteomic, or other surrogate markers of disease activity). Topiramate, an FDA-approved drug for treating epilepsy, was identified to be a potential therapeutic drug in IBD with experimental validation in a mouse model<sup>[70]</sup>. The potential role of topiramate, however, was later refuted by a retrospective cohort study<sup>[71]</sup>, and no further studies have been conducted.

As discussed previously, some diseases may not be coded in the electronic database. As an example, the effects of anti-TNF *versus* vedolizumab on arthralgia in IBD patients were studied using NLP<sup>[20]</sup>. As the electronic coding of arthralgia is not commonly performed in gastroenterology practices, Cai *et al*<sup>[20]</sup> used NLP to directly extract this non-structured information from the narrative electronic medical records, and converted it into a structured variable (joint pain: yes/no) of analysis. Without NLP, simply relying on a diagnosis code may bias any potential positive association towards null. On the other hand, manual review of the electronic medical records demands an intensive input of manpower, and accuracy is also not fully guaranteed.

In a study that involved 827,239 children, antibiotics exposure during pregnancy was found to be associated with an increased risk of very early onset IBD<sup>[72]</sup>. This study was achieved by merging data from several databases with the unique personal identity number assigned to Swedish residents. One of the databases, the Swedish Medical Birth Register, enabled the identification of child-mother links. This study illustrates the unique role of Big Data applications in investigating childhood exposure that affects disease development in adulthood, which is nearly impossible in the setting of RCT (ethical and resource issue) and other types of observational studies (*e.g.*, recall bias, resource issue).

### CRC

CRC is the third most common cancer and the second leading cause of cancer-related death<sup>[39]</sup>. As a period of 10 years is required for the development of the adenoma-carcinoma sequence<sup>[73]</sup>, identification of risk factors of CRC would have been difficult with RCTs. A large number of high-quality research has been conducted based on the NHS, NHSII and HPFS cohorts. Type II diabetes mellitus was associated with a 1.4-fold increase in CRC risk<sup>[74]</sup>. A positive association between obesity and early-onset CRC also existed among women<sup>[75]</sup>. Some of the risk factors (*e.g.*, smoking, body mass index, alcohol intake) and protective factors (*e.g.*, physical activity, folate and calcium intake) of CRC were found to be associated with the development of its precursors, adenomas and/or serrated polyps<sup>[76]</sup>. Among non-metastatic CRC patients, higher

**Table 4** Examples of studies on gastrointestinal bleeding and/or proton pump inhibitor research by utilization of large healthcare datasets

Gastrointestinal bleeding and/or proton pump inhibitors					
Country/Region	Database	Area of research	Sample size	Design, statistical methods and 3V	Application
Taiwan, China	Taiwan National Health Insurance Database (NHID)	PUD Wu <i>et al</i> <sup>[58]</sup> , 2009	403567	Nationwide retrospective cohort study  Volume, Velocity and Variety	Effect of <i>H. pylori</i> therapy and PPIs on PUD
		PUD Wu <i>et al</i> <sup>[95]</sup> , 2011	32235	Nationwide retrospective cohort study  Volume, Velocity and Variety	Risk of rebleeding from PUD in ESRD patients
		PPIs Wu <i>et al</i> <sup>[59]</sup> , 2010	6552	Nationwide retrospective cohort study  Volume, Velocity and Variety	Effect of clopidogrel and PPIs on ACS
South Korea	Korean Health Insurance Review and Assessment Service (HIRA)	PPIs Kim <i>et al</i> <sup>[96]</sup> , 2019	59233	Nationwide retrospective cohort study  Volume, Velocity and Variety	Effect of PPIs on thrombotic risk
Hong Kong, China	Clinical Data Analysis and Reporting System (CDARS)	Dabigatran Chan <i>et al</i> <sup>[62]</sup> , 2015	5041	Territory-wide retrospective cohort study  Volume, Velocity and Variety	Risk factors for dabigatran-associated gastrointestinal bleeding

This list is not exhaustive, but serves to provide a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. 3V: Volume/velocity/variety; PUD: Peptic ulcer disease; *H. pylori*: *Helicobacter pylori*; PPIs: Proton pump inhibitors; ESRD: End-stage renal disease; ACS: Acute coronary syndrome.

coffee<sup>[77]</sup>, calcium<sup>[78]</sup> and fibre<sup>[79]</sup> intake were found to be associated with a lower CRC-specific and all-cause mortality.

Concerning hereditary cancer syndromes, the Dutch Lynch syndrome Registry is one eminent example of the hereditary cancer registries. It was noted that surveillance could reduce CRC-related mortality<sup>[80]</sup>. However, in a subsequent study involving three countries (the Netherlands, Germany and Finland) with different surveillance policies, a shorter surveillance colonoscopy interval (annually) was not associated with a reduction in CRC when compared with longer intervals (1-2 yearly and 2-3 yearly intervals)<sup>[81]</sup>. The Dutch polyposis registry is another example that includes adenomatous polyposis coli patients<sup>[82]</sup>.

### HCC

Chronic hepatitis B virus (HBV) infection is a major public health threat that results in significant morbidity and mortality<sup>[83]</sup>. The prevalence of chronic HBV infection was estimated at 3.5% (257 million people) worldwide in 2016. Major complications of chronic HBV infection included HBV reactivation with hepatitis flare<sup>[84]</sup>, cirrhosis and HCC<sup>[85,86]</sup>.

Nucleos(t)ide analogue (NA) therapy was found to be associated with a lower HCC risk among chronic hepatitis B (CHB) patients<sup>[87]</sup>. This was in line with the finding from an ecologic study showing that NA therapy was associated with a reduction in age-adjusted liver cancer incidence<sup>[88]</sup>. The beneficial effect of NA was further proven among CHB patients who had undergone liver resection for HCC, in which NA therapy was associated with a lower risk of HCC recurrence<sup>[89]</sup>. The recent finding that tenofovir was associated with around a 40% reduction in HCC risk compared with entecavir has guided the choice of antiviral therapy in CHB patients at high risk of HCC (*e.g.*, cirrhosis)<sup>[90]</sup>. Although diabetes mellitus was associated with an increased HCC risk<sup>[91]</sup>, each incremental year increase in metformin use resulted in a 7% reduction in HCC risk for diabetic patients.

The choices of therapeutics drugs for HCC are still currently limited. Big Data



Table 5 Examples of studies on inflammatory bowel disease research by utilization of large healthcare datasets

Inflammatory bowel disease					
Country/Region	Database	Area of research	Sample size	Design, statistical methods and 3V	Application
South Korea	Korean Health Insurance Review and Assessment Service (HIRA)	UC Song <i>et al</i> <sup>[97]</sup> , 2018	11233	Nationwide retrospective cohort study  Comparator: general population  Volume, Velocity and Variety	Incidence and clinical impact of perianal disease in UC
Taiwan, China	Taiwan National Health Insurance Database (NHID)	IBD Chang <i>et al</i> <sup>[98]</sup> , 2018	38039	Nationwide retrospective cohort study to compare IBD patients with general population to derive SIR  Hospital based nested case-control study  Volume, Velocity and Variety	Association between IBD and herpes zoster infection
Sweden	Swedish Patient Registry	UC Myrelid <i>et al</i> <sup>[99]</sup> , 2017	63711	Nationwide retrospective cohort study  Volume, Velocity and Variety	Association between appendectomy and UC
	Swedish Medical Birth Register (child-mother link)  Swedish Multigeneration Register (child-father link)  Swedish Prescribed Drug Register National Patient Register	IBD Ortqvist <i>et al</i> <sup>[72]</sup> , 2019	827,239 children born between 2006 and 2013	Nationwide prospective population-based register study  Volume, Velocity and Variety	Association between maternal exposure to antibiotics during pregnancy and very early onset IBD in adulthood
United States	NCBI Gene Expression Omnibus (GEO)	IBD Dudley <i>et al</i> <sup>[70]</sup> , 2011	Not applicable	Signature inversion study  Volume, Velocity and Variety	Topiramate as a potential therapeutic agent against IBD
United States	Not applicable	IBD Cai <i>et al</i> <sup>[20]</sup> , 2018	1585	Retrospective cohort study Natural language processing  Volume, Velocity and Variety	Association between arthralgia and biologics (anti-TNF <i>vs</i> vedolizumab)
Not applicable	International IBD Genetics Consortium's Immunochip project	IBD Wei <i>et al</i> <sup>[64]</sup> , 2013	53279	Machine learning algorithm  Volume, Velocity and Variety	Predictors of IBD
United States	Not applicable	IBD Hou <i>et al</i> <sup>[100]</sup> , 2013	575 colonoscopy reports	Retrospective cohort study Natural language processing  Volume, Velocity and Variety	Differentiation of surveillance from non-surveillance colonoscopy
United States	Not applicable	IBD Waljee <i>et al</i> <sup>[66]</sup> , 2017	1080	Retrospective cohort study  Random Forest machine learning algorithm	Prediction of IBD remission in thiopurine users
United States	Not applicable	IBD Waljee <i>et al</i> <sup>[65]</sup> , 2017	20368	Retrospective cohort study  Random Forest machine learning algorithm	Prediction of hospitalization and outpatient steroid use
Not applicable	Phase 3 clinical trial data	IBD Waljee <i>et al</i> <sup>[67]</sup> , 2018	491	Retrospective cohort study  Random Forest machine learning algorithm	Prediction of steroid-free endoscopic remission with vedolizumab in UC

This list is not exhaustive, but serves to provide a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. 3V: Volume/velocity/variety; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; SIR: Standardized incidence ratio; anti-TNF: anti-tumour necrosis factor.

approaches in drug repurposing have once again shed light on the potential anti-cancer role of some medications currently approved for other purposes. For example, Chen *et al.*<sup>[17]</sup> collected publicly available data from HCC studies on HCC-related genes, and 6,100 drug-mediated expression profiles from Connectivity Map, which is a search engine cataloguing the effects of pharmacological compounds on different cell types. By using “signature inversion” approaches, chlorpromazine and trifluoperazine were found to have anti-cancer effects on HCC. Another study using a similar computational approach unveiled the potential anti-HCC effect of prenylamine<sup>[18]</sup>.

## FUTURE PERSPECTIVE OF BIG DATA RESEARCH

Clinicians and scientists in the field of gastroenterology and hepatology should aspire to optimize the potential advantage of powerful Big Data in translating routine clinically-collected data into precision medicine, the development of new biomarkers, and therapeutic agents in a relatively short and effective manner for preventing diseases and/or improving patient outcomes. However, some areas are still primitive or under-explored.

Parent-child linkage is one of the examples unique to Big Data analysis. Parental factors could have important bearings on the development of various diseases during childhood. One example is linking racial/ethnic and socioeconomic data from both parents with childhood obesity<sup>[92]</sup>. As for gastrointestinal and liver diseases, one study showed that maternal use of antibiotics during pregnancy was associated with an increased risk of very early onset IBD<sup>[72]</sup>. One possible mechanism is *via* the alteration of the gut microbiome<sup>[93]</sup>. However, the unavailability of direct linkage is still a major issue that can only be partly addressed by indirect inference, such as a probabilistic linkage of maternal and baby healthcare characteristics<sup>[94]</sup>. It is therefore imperative to have a database system that has direct parent-child linkages, of which many of the currently existing electronic databases are still devoid.

Drug safety is another field that could benefit from Big Data research. First, preclinical computational exclusion of potentially toxic drugs will improve patient safety while reducing the delay in drug discovery and expense. Second, the efficiency of post-marketing surveillance on drug toxicities can be enhanced. Concerning the missing data for some important risk factors (*e.g.*, smoking, alcohol intake, body mass index), administering institutions should be aware of the immense potential of Big Data, and take pre-emptive actions to start collecting these data. Although the hypothesis-free approach of Big Data analysis facilitates the discovery of new biomarkers and drugs, the results should still be validated in multi-centres. A network involving multiple centres across nations should be established to foster a centralized, comprehensive collection and validation of data. While patient privacy should be upheld, regulatory mechanisms should be realistically enforced without jeopardizing the conduct of Big Data research.

## CONCLUSION

The advent of Big Data analysis in medical research has revolutionized the traditional hypothesis-driven approach. Big Data analysis provides an invaluable opportunity to improve individual and public health. Data fusion of different sources will enable the analysis of health data from different perspectives across different regions. In this era of digitalized healthcare research and resources, manpower and time are no longer hurdles to the production of high-quality clinical studies in a cost-effective manner. With continuous technological advancements, some of the current limitations with Big Data may be further minimized.

**Table 6 Examples of studies on colorectal cancer research by utilization of large healthcare datasets**

Colorectal cancer					
Country/Region	Database	Area of research	Sample size	Design, statistical methods and 3V	Application
Hong Kong, China	Clinical Data Analysis and Reporting System (CDARS)	CRC Cheung <i>et al</i> <sup>[101]</sup> , 2019	197902	Territory-wide retrospective cohort study Volume, Velocity and Variety	Epidemiology, characteristics, risk factors and prognosis of postcolonoscopy Colorectal cancer in Asians
		CRC Cheung <i>et al</i> <sup>[69]</sup> , 2019	187897	Territory-wide retrospective cohort study PS matching Volume, Velocity and Variety	Association between statins and CRC
United States	Nurses' Health Study II (NHSII)	CRC Ma <i>et al</i> <sup>[74]</sup> , 2018	134763	Prospective cohort study Volume and Variety	Association between DM and CRC
	Health Professionals Follow-up Study (HPFS)				
	Nurses' Health Study (NHS)	CRC Yang <i>et al</i> <sup>[78]</sup> , 2018	1660	Prospective cohort study	Effect of calcium intake, coffee and fibre on survival after CRC diagnosis
	Health Professionals Follow-up Study (HPFS)	Hu <i>et al</i> <sup>[77]</sup> , 2018 Song <i>et al</i> <sup>[79]</sup> , 2018	1599 1575	Volume and Variety	
	Nurses' Health Study (NHS)	CRC He <i>et al</i> <sup>[76]</sup> , 2018	141143	Prospective cohort study	Risk factors of serrated polyps and conventional adenomas
	Nurses' Health Study II (NHSII)	de Jong <i>et al</i> <sup>[80]</sup> , 2006		Volume and Variety	
	Health Professionals Follow-up Study (HPFS)				
Netherlands	Dutch Lynch syndrome Registry	Various cancers including CRC	2788	Retrospective cohort study Volume, Velocity and Variety	Decrease in CRC-related mortality in Lynch syndrome families by surveillance
Netherlands, Germany, Finland	Dutch Lynch syndrome Registry	CRC Engel <i>et al</i> <sup>[81]</sup> , 2018	2747 patients with 16327 colonoscopies	Retrospective cohort study	Surveillance interval on CRC incidence and stage
	German HNPCC Consortium Finland			Volume, Velocity and Variety	

This list is not exhaustive, but serves to provide a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. 3V: Volume/velocity/variety; CRC: Colorectal cancer; DM: Diabetes mellitus.

**Table 7 Examples of studies on hepatocellular carcinoma research by utilization of large healthcare datasets**

Hepatocellular carcinoma					
Country/Region	Database	Area of research	Sample size	Design, statistical methods and 3V	Application
Taiwan, China	Publicly available data on HCC-related genes Connectivity Map (CMap) -- includes 6100 drug-mediated expression profiles	HCC Chen <i>et al</i> <sup>[17]</sup> , 2011	Not applicable	Signature inversion study Volume, Velocity and Variety	Anti-cancer effects of chlorpromazine and trifluoperazine on HCC

	Taiwan National Health Insurance Database (NHID)	HCC Wu <i>et al</i> <sup>[89]</sup> , 2012	4569	Nationwide retrospective cohort study Volume, Velocity and Variety	Association between NA therapy and HCC recurrence among patients with HBV-related HCC after liver resection
	Taiwan National Health Insurance Database (NHID)	HCC Chen <i>et al</i> <sup>[91]</sup> , 2013	292290	Nationwide case-control study Volume, Velocity and Variety	Association between DM and HCC
	Taiwan National Health Insurance Database (NHID)	HCC Wu <i>et al</i> <sup>[87]</sup> , 2014	43190	Nationwide retrospective cohort study PS matching Volume, Velocity and Variety	Association between NA therapy and HCC among CHB patients
China	The Cancer Genome Atlas (TCGA) database Connectivity Map (CMap)	HCC Wang <i>et al</i> <sup>[18]</sup> , 2016	Not applicable	Signature inversion study Volume, Velocity and Variety	Anti-cancer effect of prenylamine on HCC
South Korea	Korean Health Insurance Review and Assessment Service (HIRA)	HCC Choi <i>et al</i> <sup>[90]</sup> , 2018	24156	Nationwide retrospective cohort study Volume, Velocity and Variety	Difference between tenofovir and entecavir on reducing HCC risk
Hong Kong, China	Clinical Data Analysis and Reporting System (CDARS)	HCC Seto <i>et al</i> <sup>[88]</sup> , 2017	Entire Hong Kong population between 1999 and 2012	Territory-wide retrospective cohort study Volume, Velocity and Variety	Association between NA therapy and HCC among CHB patients
Sweden	Swedish Cancer Registry Swedish Patient Registry	HCC Ji <i>et al</i> <sup>[102]</sup> , 2012	9160 CHB patients	Nationwide retrospective cohort study Comparison with general population to derive SIR Volume, Velocity and Variety	Association between concomitant HBV/HDV infection and HCC

This list is not exhaustive, but serves to provide a few distinct examples of how Big Data analysis can generate high-quality research outputs in the field of gastroenterology and hepatology. 3V: Volume/velocity/variety; HCC: Hepatocellular carcinoma; NA: Nucleos(t)ide analogue; DM: Diabetes mellitus; PS: Propensity score; CHB: Chronic hepatitis B; SIR: Standardized incidence ratio; HDV: Hepatitis D virus.

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## Biomarkers and subtypes of deranged lipid metabolism in non-alcoholic fatty liver disease

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous and complex disease that is imprecisely diagnosed by liver biopsy. NAFLD covers a spectrum that ranges from simple steatosis, nonalcoholic steatohepatitis (NASH) with varying degrees of fibrosis, to cirrhosis, which is a major risk factor for hepatocellular carcinoma. Lifestyle and eating habit changes during the last century have made NAFLD the most common liver disease linked to obesity, type 2 diabetes mellitus and dyslipidemia, with a global prevalence of 25%. NAFLD arises when the uptake of fatty acids (FA) and triglycerides (TG) from circulation and de novo lipogenesis saturate the rate of FA  $\beta$ -oxidation and very-low density lipoprotein (VLDL)-TG export. Deranged lipid metabolism is also associated with NAFLD progression from steatosis to NASH, and therefore, alterations in liver and serum lipidomic signatures are good indicators of the disease's development and progression. This review focuses on the importance of the classification of NAFLD patients into different subtypes, corresponding to the main alteration(s) in the major pathways that regulate FA homeostasis leading, in each case, to the initiation and progression of NASH. This concept also supports the targeted intervention as a key approach to maximize therapeutic efficacy and opens the door to the development of precise NASH treatments.

**Key words:** S-adenosylmethionine; Methionine adenosyltransferase; Lipid metabolism; Multiomics; Lipidomics; Nonalcoholic steatohepatitis; One-carbon metabolism; Very low-density lipoproteins; Steatosis; Precision medicine

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**Core tip:** Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous and complex disease that is imprecisely diagnosed by liver biopsy. The advent of metabolomics has shown that NAFLD progression from simple steatosis to nonalcoholic steatohepatitis (NASH) associates with profound alterations in liver and serum lipidomic signatures that are good indicators of the disease's development and progression. Lipidomics has also permitted the classification of NAFLD patients into different subtypes corresponding to the main alteration(s) leading, in each case, to the initiation and progression of NASH based on the identification of specific lipid signatures, opening the door to the development of precise NASH treatments.

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## INTRODUCTION

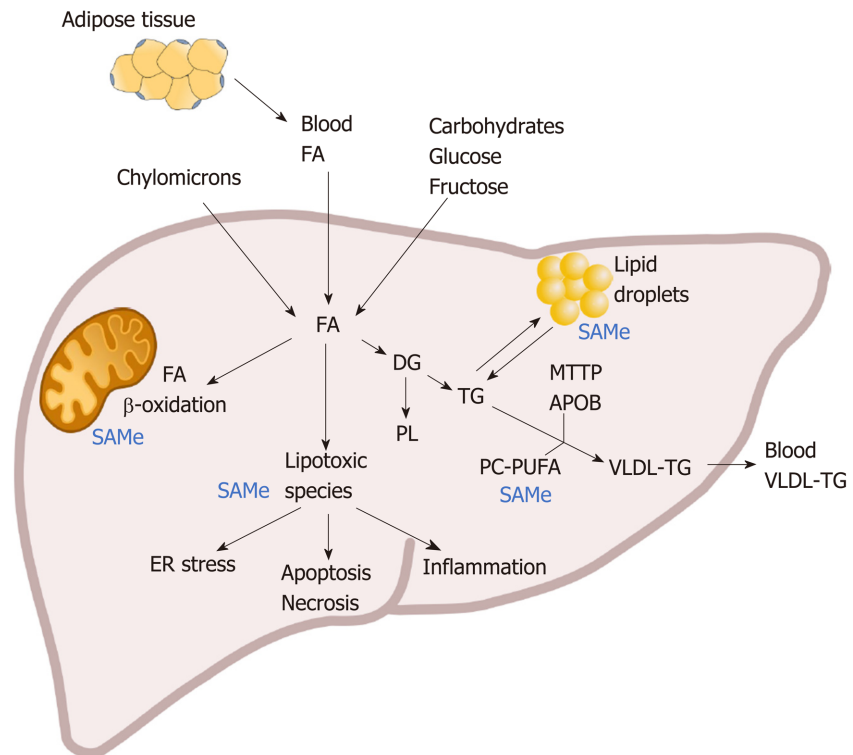
Fat storing is common to many different species. The desert locust stores lipids in the "fat body", a dynamic tissue that plays an essential role in energy storage and utilization in insects<sup>[1]</sup>, to migrate from south-western Morocco to the Iberian Peninsula covering a distance of 600 miles without settling down. Some fish also store fat for their survival. Without eating and powered only by stored fat, salmon swim 2000 miles up the fresh waters of the Yukon River from the Bering Sea to reach their spawning grounds. Long distance migrating birds, such as the bar-tailed godwit, the ruby-throated hummingbirds and the bar-headed geese, accumulate large amounts of fat prior to departing. Likewise, the gray whale increases its fat stores prior to swimming more than 10000 miles between feeding grounds in the Arctic to the nursery lagoons of Mexico's Baja Peninsula; and hibernating mammals such as the grizzly bears, after a period of incomparable hyperphagia, do not eat for 5 to 7 mo subsisting solely on stored fat.

The energy source for these prodigious feats are fatty acids (FA) stored as triglycerides (TG) into lipid droplets (LD) primarily in the adipose tissue and liver. The mobilization of FA from adipose tissue TG stores requires the activity of TG lipases that generate FA, which are then released into the blood and taken up by hepatocytes, where are reincorporated into TG (Figure 1). Some of these re-esterified TG combine with apolipoprotein-B (APOB) to form very low-density lipoproteins (VLDL), and are exported into circulation. This process is regulated by microsomal TG transfer protein (MTTP) and accompanied by encapsulating the neutral lipid core with a phospholipid (PL) monolayer enriched in phosphatidylcholine (PC) molecules containing polyunsaturated FA (PUFA), such as arachidonic acid (AA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3)<sup>[2,3]</sup>. APOB, cholesterol and other apolipoproteins (like APOC) are also found decorating the surface of the VLDL-TG particle<sup>[2,3]</sup>. The largest amount of TG used for the synthesis of VLDL (VLDL-TG) is synthesized from FA entering the liver from the adipose tissue, even under conditions where the synthesis of FA from glucose and fructose by *de novo* lipogenesis (DNL) is high (see below). Humans preferentially oxidize carbohydrate over fat, a process that helps to maintain blood glucose homeostasis. Most of the TG in circulation during the post-absorptive phase are associated with VLDL-TG<sup>[2]</sup>. This mechanism uncouples hepatic TG synthesis (energy storing) from TG secretion and maintains a low blood content of FA, which are cytotoxic.

## NONALCOHOLIC FATTY LIVER DISEASE

TG are energy dense and chemical stable compounds. By weight, FA provide more than twice as much energy (9 kcal/g) as carbohydrates and proteins (4 kcal/g), and match the caloric density of diesel (8 kcal/g). From this perspective, fatty liver may be considered a physiological adaptation and an evolutionary advantage to anticipate periods of prolonged food (energy) shortage. However, lifestyle and eating habit changes during the last century have made fatty liver the most common liver disease





**Figure 1 Lipid metabolism.** The mobilization of fatty acids (FA) from their triglyceride (TG) storage in the adipose tissue is promoted by TG lipases. The resultant FA are then released into the blood and taken up by hepatocytes. Other sources of hepatic FA are the dietary lipids in chylomicrons and *de novo* lipogenesis induced by carbohydrates. These FA are metabolized by mitochondrial or peroxisomal  $\beta$ -oxidation, accumulated in the cytoplasm inducing lipotoxicity, or subsequently elongated, desaturated and re-esterified for synthesis of complex lipids such as phospholipids (PL), diglycerides or TG. Some of the re-esterified TG are packed into very low-density lipoproteins combined with apolipoprotein-B and exported into circulation. This process is regulated by microsomal triglyceride transfer protein and accompanied by encapsulating the neutral lipid core with a PL monolayer enriched in phosphatidylcholine molecules containing polyunsaturated FA. Enzyme reactions regulated by S-adenosylmethionine (SAMe) and pathways in which SAMe deficiency may lead to the accumulation of TG and progression to nonalcoholic steatohepatitis are indicated in blue. APOB: Apolipoprotein-B; DG: Diglycerides; ER: Endoplasmic reticulum; FA: Fatty acids; MTTP: Microsomal triglycerides transfer protein; PC-PUFA: Phosphatidylcholines containing polyunsaturated fatty acids; PL: Phospholipids; SAMe: S-adenosylmethionine; TG: Triglycerides; VLDL: Very low-density lipoproteins.

linked to obesity, type 2 diabetes mellitus (T2D) and dyslipidemia, with a prevalence of 25%<sup>[4-7]</sup>. Nonalcoholic fatty liver disease (NAFLD) covers a spectrum that ranges from simple steatosis (NAFL), nonalcoholic steatohepatitis (NASH) with varying degrees of fibrosis, to cirrhosis, which is a major risk factor for hepatocellular carcinoma (HCC). NASH is distinguished from steatosis by the presence of inflammation and hepatocyte injury. Approximately 25% of individuals with NAFL progress to NASH. Of those that develop NASH, 25% progress to cirrhosis, of whom at least 1%-2% per year develop HCC<sup>[4-6]</sup>. NASH is now the leading cause of liver transplantation in women<sup>[8]</sup> and projected to be the leading indication in the United States by 2020<sup>[4-6]</sup>. Degree of liver fibrosis is the major factor linked to all-cause mortality<sup>[9]</sup>. However, NAFLD does not always follow an orderly progression. For instance, it is possible for NAFLD patients to develop fibrosis without going through the NASH stage, or to develop liver cancer despite absence of fibrosis or histologic NASH<sup>[4-6,10]</sup>. Studies have reported 10-70% of HCC cases in NAFLD occurred without cirrhosis<sup>[11]</sup>. The annual direct medical cost is > \$100 billion in the United States alone for NAFLD<sup>[4-6]</sup>. Despite the huge investment by the pharmaceutical industry there are still no approved therapies targeting NASH<sup>[12]</sup>. Lifestyle changes are the only therapeutic strategy that can halt the progression of NAFLD<sup>[4-6]</sup>. Clearly, both a better understanding of the factors that promote progression from simple steatosis to NASH, fibrosis and liver cancer is sorely needed to improve our therapeutic strategy.

## LIVER LIPID METABOLISM IN NAFLD

Consisting with its energy storage function, the relationship between the intrahepatic TG (IHTG) content and VLDL-TG secretion rate is curvilinear. In subjects with normal IHTG (up to 5% of liver weight), VLDL-TG export increases linearly with IHTG content; but in individuals with steatosis, VLDL-TG secretion reaches a plateau independently of the amount of IHTG<sup>[13,14]</sup>. Genetic defects (*APOB*, *APOC3*, *MTTP*, *TM6SF2*) that impair hepatic VLDL-TG secretion cause hepatic steatosis that may progress to NASH with fibrosis, even without obesity or T2D<sup>[15-19]</sup>; and impaired *APOB* synthesis has been observed in NASH patients as compared to obese controls<sup>[20]</sup>. These results indicate that a reduction in the capacity to export VLDL-TG increases the risk to develop NASH. Consistently, patients treated with antisense *APOB* or *MTTP* inhibitors, which lower VLDL assembly and secretion, are associated with hepatic steatosis, inflammation and fibrosis, which limit their utility<sup>[21,22]</sup>. The discovery that the effect of defective VLDL-TG secretion extends well beyond the management of liver energy storage to promote the development of NASH and fibrogenesis emphasizes the importance of identifying therapeutic targets for NASH reversal in the setting of impaired VLDL-TG secretion. It is important to note, however, that the increase in susceptibility to develop NASH in obese subjects that are carriers of the *TM6SF2* E167K variant, which impairs VLDL-TG export, is accompanied by protection from cardiovascular disease due to the reduced serum levels of atherogenic lipoproteins<sup>[23]</sup>. This is important when designing treatments that aim to increase VLDL-TG export in NASH.

Hepatic steatosis arises when the uptake of FA and TG from circulation and DNL saturate the rate of FA  $\beta$ -oxidation (in the mitochondria and peroxisomes) and VLDL-TG export (Figure 1). NAFLD subjects often show an increase in DNL<sup>[13,24]</sup>, and it has been proposed by many that DNL is a major pathway in the pathogenesis of NAFLD<sup>[25]</sup>. On this premise the pharmacological inhibition of DNL that include (1) Downregulating SREBP-1c, the major transcriptional regulator of the enzymes involved in DNL, (2) Decreasing the activity of the DNL rate-limiting enzyme, specifically acetyl-CoA carboxylase (ACC), and (3) Inhibiting stearoyl-CoA dehydrogenase 1 (SCD1), the first irreversible step committing FA to TG synthesis, are being studied in phase 2 and 3 clinical trials of NASH<sup>[26]</sup>. However, a potential limitation of this approach is that a decrease in DNL may induce an increase in FA uptake to the liver from circulation, the major source of hepatic lipids, or a decrease in FA oxidation as compensatory mechanisms<sup>[27,28]</sup>. From an evolutionary stand point, it seems unlikely that an increase in DNL would be a major pathway in the development of NAFLD. FA from the adipose tissue and from the diet contribute about 59% of TG in the livers of patients with NAFLD, while DNL contributes 26% of intrahepatic FA, and dietary TG transported by chylomicrons 15% of liver fat<sup>[29]</sup>. Accordingly, the inhibition of liver FA uptake has been shown to improve NASH in experimental models<sup>[30]</sup>; albeit at the risk of increasing FA in circulation, peripheral FA stores, and weight gain, which may limit its potential therapeutic application. The importance of increased DNL in NASH development should, however, not be minimized since increased DNL may just as well overwhelm a deficient VLDL-TG exporting system which, presumably, is already saturated caused by increased hepatocellular lipid uptake. The increase in DNL in NAFLD may be an adaptive mechanism for the generation of metabolic signals that direct lipids toward beneficial pathways to improve energy balance even in the setting of excess FA accumulation, a concept known as lipoexpediency (the antonym to lipotoxicity<sup>[31,32]</sup>). For instance, it has been shown that FA synthase, the DNL enzyme that catalyzes the conversion of acetyl-CoA to the 16-carbon FA palmitate, is involved in the activation of PPAR $\alpha$  (an activator of FA oxidation that is expressed at high concentrations in the liver) *via* the synthesis of its ligand, palmitoyl-stearoyl-phosphatidylcholine (PC-16:0/18:1)<sup>[33]</sup>. NAFLD subjects also show an increase in the rate of hepatic FA oxidation<sup>[34,35]</sup> because of mitochondrial uncoupling between FA oxidation and ATP synthesis<sup>[36]</sup>. Increased FA oxidation in NAFLD may be, however, detrimental to the liver due to the excessive generation of reactive oxygen species. Together, these results suggest that different individuals (NAFLD subtypes) could have different alterations in the major pathway(s) that regulate FA homeostasis leading to NAFLD<sup>[37]</sup>. Evidence from clinical trials indicating that only a small percent (20%-50%) of NASH patients benefit from the different treatments supports this concept<sup>[26]</sup>. Thus, the identification of noninvasive metabolic biomarkers that would allow the classification of patients into different subtypes that correspond to the main alteration(s) leading to the initiation and progression of NASH would be of great help for the development of precise treatments.

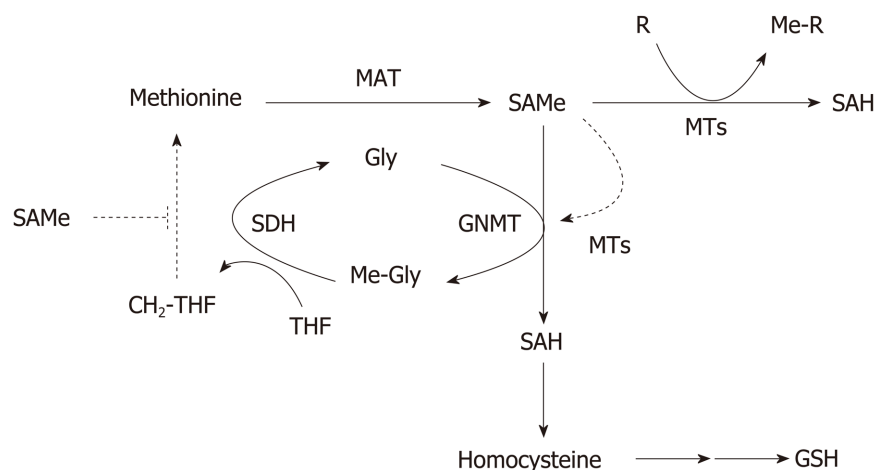
## S-ADENOSYLMETHIONINE AS A LINK BETWEEN LIPID METABOLISM AND HEPATOCELLULAR ONE CARBON METABOLISM

Assessing the hepatic lipid metabolism, it is important to note that LD are not only critically important for energy metabolism in terms of TG storage, but are also a major supply of (1) PL precursors, such as diacylglycerols (DG) and other lipids of the monoalk(en)yl diacylglycerol family, that give rise to diacyl-PL and plasmalogens, respectively; (2) Cholesterol, which is stored as cholesteryl-esters (CE); and (3) FA, not only saturated FA, such as palmitate (16:0) and stearate (18:0), which are cytotoxic, but also PUFA, such as AA, that gives rise to the eicosanoid family of inflammatory mediators (prostaglandins, thromboxanes, and leukotrienes), and DHA, which is anti-inflammatory<sup>[38]</sup>. The main lipid classes found in the core of liver LD are TG, DG, and CE, which are enveloped by a PL monolayer (mainly made of PC) decorated with proteins that are important in lipid remodeling, signaling and energy storing<sup>[39,40]</sup>. PC found in LD are synthesized both by the Kennedy route, whose last step is the reaction of CDP-choline with DG to form PC and cytidine monophosphate; and the PE *N*-methyltransferase (PEMT) pathway, which converts PE rich in PUFA (mainly AA and DHA) into PC through three successive *N*-methylations of the PE amino group, with S-adenosylmethionine (SAME) as the methyl donor<sup>[41]</sup> (Figure 1). SAME is a versatile molecule which is the source of essentially all methyl transfer reactions in cells<sup>[42]</sup>. Liver plays a central role in SAME metabolism, as this is where up to half of the daily intake of methionine is catabolized *via* its conversion to SAME<sup>[43]</sup>. This reaction is catalyzed by methionine adenosyltransferase (MAT). Two genes encode for MAT, *MAT1A* is expressed in normal differentiated liver and *MAT2A* is expressed in all extrahepatic tissues as well as in fetal liver<sup>[43]</sup>. In liver, SAME homeostasis is controlled by MAT-mediated synthesis and utilization, largely accomplished by glycine *N*-methyltransferase (GNMT)<sup>[43]</sup> (Figure 2). Accordingly, GNMT deletion in mice induces a massive increase in intrahepatic SAME content<sup>[44]</sup> that accelerates the flux of methyl groups through multiple pathways, including PEMT and DNA-methylation, leading to aberrant liver lipid signatures, development of NASH, fibrosis and HCC<sup>[45]</sup>.

SAME metabolism is coupled to the folate cycle and together they form the so called one carbon metabolism (1CM) (Figure 3). 1CM circulates 1-carbon units from different nutritional and amino acids inputs (choline, betaine, folate, glucose, methionine, serine, glycine and threonine), *via* SAME and 5-methyltetrahydrofolate (MTHF), into a large variety of outputs, such as PL-, protein- and DNA-methylation, and glutathione (GSH), polyamines, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and nucleotide synthesis, that regulate key biological processes ranging from VLDL-TG export, gene expression and redox homeostasis, to DNA synthesis and cell growth. *Mat1a* knockout (KO) mice have chronically low hepatic SAME level (75% lower)<sup>[46]</sup>, show reduced content of PC-PUFA (mainly AA and DHA)<sup>[37]</sup> and, as expected, impaired synthesis and release of VLDL-TG, which leads to the accumulation of TG, DG and FA, accumulation of oxidized FA, oxidative stress, and abnormal hepatic lipid signatures, which trigger the spontaneous development of steatosis and its progression to NASH, fibrosis and HCC<sup>[37,43,46]</sup>. In *Mat1a* KO mice, low SAME also associates with increased serum levels of amino acids methionine, serine and glycine; increased hepatic MTHF, decreased GSH content, and altered protein and DNA methylation<sup>[37]</sup>. *MAT1A* is often downregulated in NAFLD patients with more advanced fibrosis<sup>[47]</sup>. Consistently, several studies showed human NASH have reduced transmethylation<sup>[48]</sup>, hepatic PC/PE ratio<sup>[49]</sup>, and abnormal VLDL-TG assembly and export<sup>[50]</sup>. These results suggest that SAME deficiency may be a critical driver of NASH in a subgroup of NAFLD patients. Importantly, SAME treatment of the *Mat1a* KO mice after onset of NASH for two months corrected many of the abnormalities, nearly normalized the liver histology, and reduced blood ALT, AST and TG levels without altering cholesterol content<sup>[37]</sup>. SAME treatment of rats fed a methionine and choline deficient (MCD) diet, which reduces hepatic SAME content and induces steatohepatitis, also improved liver histology<sup>[51]</sup>. Taken together, these results support the concept that 1) a reduction in SAME is a common driver of NAFLD initiation and progression to NASH in humans, and 2) that NAFLD patients with M-subtype serum metabolomic profile (see below) will likely benefit from SAME treatment, but this has not yet been examined.

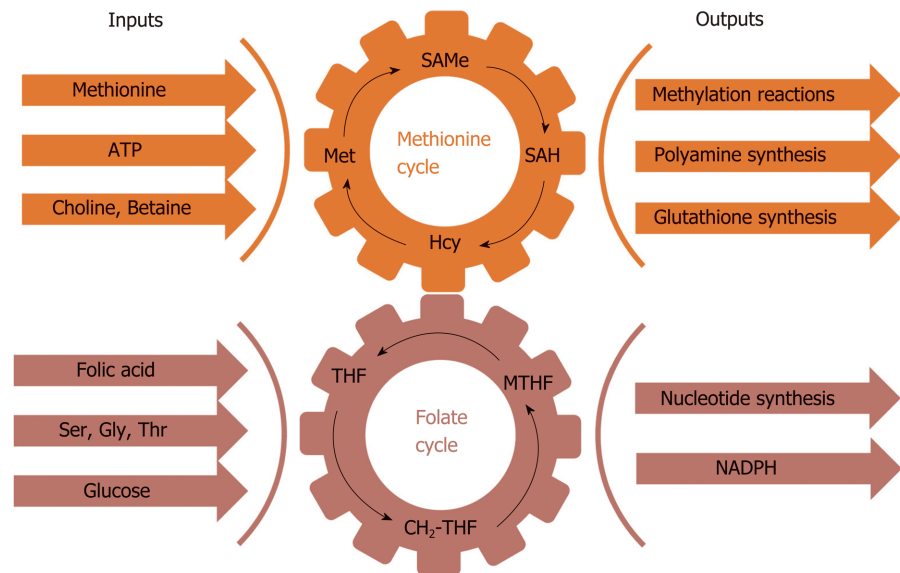
## CIRCULATING BIOMARKERS OF NAFLD

The advent of lipidomics has taught us that each lipid class (*e.g.*, TG, PC) is made of a



**Figure 2 Regulation of hepatic S-adenosylmethionine homeostasis.** Hepatic S-adenosylmethionine (SAME) content is regulated by the concerted activity of methionine adenosyltransferase (MAT) and glycine *N*-methyltransferase (GNMT). Methionine is mainly metabolized by the liver where is converted to SAME by the enzyme MAT using ATP as co-substrate. SAME, the main cellular methyl donor, is converted to S-adenosylhomocysteine (SAH) by a legion of methyltransferases (MTs) that catalyze the methylation of multiple substrates (DNA, proteins, phospholipids, small molecules, toxic and waist products). Excess SAME is catabolized by GNMT, the most abundant hepatic MT, to prevent undesirable methylations. The GNMT-sarcosine dehydrogenase (SDH) pathway recycles the excess of methyl groups *via* generation of methylene-tetrahydrofolate (CH<sub>2</sub>-THF) and the methylation of homocysteine to regenerate methionine (not shown) to maintain SAME homeostasis. SAH is converted to homocysteine, a metabolic crossroad that can be used for the regeneration of methionine (not shown) or the synthesis of glutathione depending on whether the concentration of SAME is low or high, respectively. SAME is an allosteric activator of GNMT and an inhibitor of the re-synthesis of methionine *via* the CH<sub>2</sub>-THF pathway (broken lines). CH<sub>2</sub>-THF: 5,10-methylene-tetrahydrofolate; Gly: Glycine; GNMT: Glycine *N*-methyltransferase; MAT: Methionine adenosyltransferase; Me-Gly: Methylglycine (sarcosine); Me-R: Methylated product; MTs: Methyltransferases; MTHF: 5-methyltetrahydrofolate; R: Methylation substrate; SAH: S-adenosylhomocysteine; SAME: S-adenosylmethionine; SDH: Sarcosine dehydrogenase; THF: Tetrahydrofolate.

multitude of different lipid molecular species varying in the length and number of double bonds of their FA chains<sup>[52,53]</sup>; and that the lipid homeostatic status is implemented by a large family of FA desaturases and elongases in conjunction with lipases, acyl-transferases, PL and sphingolipid synthesizing enzymes, and phospholipases<sup>[54,55]</sup>. Changes in lipid signatures (lipid molecular species compositions) can have profound effects on cell function, regulating processes such as oxidative phosphorylation<sup>[56]</sup>. A sequence variant in PNPLA3 that is strongly associated with NAFLD has been related to TG remodeling and VLDL-TG secretion in hepatocytes<sup>[57,58]</sup>, suggesting that abnormal lipid remodeling may be key to the development and progression of NAFLD. Accordingly, mice modify the liver lipid profile in response to a variety of conditions that induce steatosis and its progression to NASH, such as ablation of methionine adenosyltransferase 1A (*Mat1a*)<sup>[46]</sup>, fasting or feeding a high fat diet<sup>[41]</sup>, or feeding an MCD diet<sup>[59]</sup>. It has also been observed that the serum lipidomic profile reflects the liver lipidome<sup>[37]</sup>, a finding which supports the search of noninvasive NAFLD biomarkers in blood. At present, liver biopsy is the “gold standard” to diagnose NASH, an invasive, imprecise and expensive procedure with possible complications. As a result, numerous studies have been published aiming to the identification of panels of circulating biomarkers (using genomics, transcriptomics, proteomics and metabolomics) for steatosis, NASH and fibrosis diagnosis, as well as for risk prediction of NAFLD progression and response to therapy<sup>[60,61]</sup>. Some studies have shown that lipidomic patterns can differentiate between normal liver and NAFLD<sup>[62,63]</sup>. Interestingly, recent studies also focus on the discrimination between simple steatosis and NASH<sup>[64]</sup> or the detection of advance fibrosis<sup>[65]</sup>. However, a burning challenge in NAFLD research is the identification of which patients with NAFLD will develop NASH and, for those with NASH, how fast the disease will progress. At present, it is premature to conclude which of these blood biomarkers, alone or in combination, would be best to precisely and rapidly diagnose the severity of NASH and monitor the liver’s response to treatment<sup>[60,61]</sup>.



**Figure 3 Schematic representation of one carbon metabolism.** One carbon metabolism involves multiple physiological processes in which one carbon units circulate from different nutritional and amino acids inputs (choline, betaine, folic acid, glucose, methionine, serine, glycine and threonine), mediated by S-adenosylmethionine and 5-methyltetrahydrofolate, and are converted into a wide variety of outputs, such as the methylation of phospholipids, protein and DNA, and the synthesis of glutathione, polyamines, nucleotides, and reduced nicotinamide adenine dinucleotide phosphate. CH<sub>2</sub>-THF: Methylene tetrahydrofolate; Gly: Glycine; GSH: Glutathione; Hcy: Homocysteine; Met: Methionine; MTHF: 5-Methyltetrahydrofolate; NADPH: Reduced nicotinamide adenine dinucleotide phosphate; SAH: S-adenosylhomocysteine; SAMe: S-adenosylmethionine; Ser: Serine; THF: Tetrahydrofolate; Thr: Threonine.

## IDENTIFICATION OF NAFLD SUBTYPES

Despite the current essential role of biopsy for NAFLD diagnosis, its use as a tool for determining the different metabolic pathways that lead to the initiation and progression of NAFLD is certainly limited. Recently, lipidomics has permitted the classification of NAFLD patients into different subtypes corresponding to the main alteration(s) leading, in each case, to the initiation and progression of NASH based on the identification of specific lipid signatures. We identified a unique serum metabolomic profile that distinguished between *Mat1a* KO and wild type (WT) mice and observed, using a large cohort of 535 serum samples from biopsied NAFLD patients, that nearly half of them showed this *Mat1a* KO-type (M-subtype) metabolomic signature<sup>[37]</sup> (Table 1). Although classification based on this approach is not indicative of disease progression (M-subtype is equally distributed among patients with steatosis and NASH), a small group of serum metabolites that could differentiate simple steatosis from NASH in the *Mat1a* KO and in NAFLD patients was also identified. This work defined, for the first time, the metabolic landscape affected by a chronically reduced hepatic SAMe level and demonstrated key abnormalities that were corrected by SAMe treatment, which led to resolution of NASH.

The MCD diet model is a widely-used murine model of NASH but animals lose weight rapidly, have low serum TG levels, and do not become insulin resistant<sup>[66]</sup>. The addition of 0.1% methionine (normal diet contains 0.3% methionine) minimizes weight loss and yet mice fed the 0.1MCD diet have low liver SAMe content and developed steatosis, inflammation and fibrosis<sup>[59]</sup>. The mechanism for steatosis included impaired VLDL-TG secretion and reduced GSH, due to the decrease in SAMe content, the concomitant reduction in the synthesis of PC-PUFA through the PEMT pathway, and increased uptake of FA *via* CD36. Despite the existence of important differences between both models [(1) The protein content of SCD1 is increased in *Mat1a* KO and decreased in 0.1MCD; and (2) Mitochondrial FA  $\beta$ -oxidation is decreased in *Mat1a* KO and increased in 0.1MCD], the reduction in hepatic SAMe content is the common driver of NAFLD initiation and progression to NASH in both of them and, accordingly, NAFLD patients classified as M-subtype were found to have a metabolic profile similar to the 0.1MCD model<sup>[59]</sup> (Table 1). Treatment of the 0.1MCD mice for two weeks, after the onset of NAFLD, with the SCD1 inhibitor arachidyl amido cholanoic acid (Aramchol, a Phase 2b test drug candidate in a clinical trial for NASH)<sup>[67]</sup>, improved the liver histology<sup>[59]</sup> (Table 1).



**Table 1** Nonalcoholic fatty liver disease subtype classification

NAFLD subtype	Characteristics	Mouse model-based classification	Treatments tested
M-Subtype	Increased fatty acid uptake.	<i>Mat1a</i> KO <sup>[37]</sup>	SAMe
	Low liver glutathione and SAMe content.	0.1MCD <sup>[59]</sup>	Aramchol
	Reduced synthesis of PC-PUFA.		
	Abnormal VLDL-TG assembly and export.		
Non-M-Subtype	Increased DNL.	<i>Ldlr</i> KO/HFD <sup>[68]</sup>	Obeticholic acid
	Normal hepatic SAMe levels.		
	Normal VLDL-TG secretion.		
	High serum levels of cholesterol and TG.		

NAFLD: Nonalcoholic fatty liver disease; SAMe: S-adenosylmethionine; PC-PUFA: Phosphatidylcholines containing polyunsaturated fatty acids; VLDL-TG: Very low-density lipoprotein-triglycerides; DNL: *de novo* lipogenesis; KO: Knockout; MCD: Methionine and choline deficient.

Aramchol has been shown to improve the three key pathologies associated to NASH: (1) Steatosis, by reducing TG synthesis and increasing VLDL-TG export and FA  $\beta$ -oxidation; (2) Inflammation, by decreasing lipotoxicity; and (3) Fibrosis, by downregulation of collagen production by stellate cells<sup>[59]</sup>. We speculate *Mat1a* KO mice, and therefore NAFLD patients with M-subtype serum metabolomic profile, will likely benefit from Aramchol treatment.

Interestingly, nearly all NAFLD patients classified as having a non-M-subtype, according to both the *Mat1a* KO and 0.1MCD metabolomics models of NASH, were found to have a lipidomic signature similar to that found in low-density lipoprotein receptor (*Ldlr*) KO mice fed a high fat diet (HFD)<sup>[68]</sup> (Table 1). This mouse model (*Ldlr* KO/HFD) shows high serum levels of cholesterol and TG, normal liver SAMe, and develop NASH and fibrosis. Treatment of the *Ldlr* KO/HFD mice for ten weeks, after the onset of NAFLD, with the Farnesoid X Receptor agonist Obeticholic acid (OCA, a Phase 3 test drug candidate in a clinical trial for NASH)<sup>[69]</sup>, nearly normalized the liver histology, reduced blood ALT, AST and TG levels and tended to lower cholesterol content<sup>[68]</sup>. It would be interesting to determine if in the Aramchol and OCA clinical trials for NASH, patients that responded to treatment were enriched in M- and non-M-subtype, respectively.

However, this approach also results in a certain number of unclassified patients (named as indeterminate)<sup>[37,59]</sup>, which can be inherently linked to the unsupervised classification methodology and validation procedure. Potential integration of other omics data as well as clinical parameters may improve this novel subtyping approach of NAFLD patients, allowing further interpretation of the complex biochemical processes and the heterogeneity of the disease.

## CONCLUSION

To understand the pathogenesis of NASH, a useful conceptual framework is that the liver's capacity to accumulate and export TG supports two crucial physiological functions (1) Storing highly energetic, but also highly cytotoxic, FA stably as TG, and (2) Placing into circulation the right amount of VLDL-TG to meet the energy needs of extrahepatic tissues. Both functions collide when the IHTG content exceeds 5% and hepatocytes must safely handle and accumulate excess FA into TG without increasing the rate of VLDL-TG export<sup>[13,14]</sup>. The maximum capacity to safely handle FA by the liver in the presence of increasing levels of IHTG may vary between individuals depending on the variable contributions from different molecular pathway(s) that result in TG accumulation; and NASH may develop when this maximum capacity is exceeded. The observation that it is possible for NAFLD patients to develop NASH at various grades of steatosis, supports this notion. However, clinical trials currently designed for the treatment of NASH are based on the mechanism of action of a drug that is administered to patients without confirming if that specific molecular pathway is altered; which is against the view that NASH pathogenesis has diverse drivers. Understandably, no more than 40% of patients in these trials have shown a positive response to treatment<sup>[26]</sup>. Alternatively, a comprehensive landscape of the main NASH drivers may be obtained, for example, by integrating multiomics data of well-defined mouse models of NASH, for which the efficacy of different drugs have been validated, with the multiomics data of a large cohort of well-characterized NASH patients following a similar procedure to that previously described<sup>[37]</sup>. Such a strategy

would associate patients' multiomics signatures to specific therapies that could be validated reanalyzing the data of clinical trials where the efficacy of these drugs has been tested. In addition, this approach may allow advances in our understanding of the complex biochemical processes and pathophysiological responses in NAFLD<sup>[70,71]</sup>. Moreover, it will be also important to integrate gene products, mRNA, proteins and metabolites, with environmental factors, such as diet and life style<sup>[72,73]</sup>. Finally, this strategy may be extended to the identification of optimal therapeutic drug combinations.

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## Imaging biomarkers for the treatment of esophageal cancer

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### Abstract

Esophageal cancer is known as one of the malignant cancers with poor prognosis. To improve the outcome, combined multimodality treatment is attempted. On the other hand, advances in genomics and other “omic” technologies are paving way to the patient-oriented treatment called “personalized” or “precision” medicine. Recent advancements of imaging techniques such as functional imaging make it possible to use imaging features as biomarker for diagnosis, treatment response, and prognosis in cancer treatment. In this review, we will discuss how we can use imaging derived tumor features as biomarker for the treatment of esophageal cancer.

**Key words:** Esophageal cancer; Computed tomography perfusion; Dynamic-contrast-enhanced magnetic resonance imaging; Texture analysis; Diffusion-weighted imaging; Positron emission tomography

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**Core tip:** Advances in imaging techniques enable us to assess tumor biology such as viability, vascular physiology, heterogeneity, or metabolism, which can be new approaches to investigate biomarker for cancer treatment. In this review, we will discuss various functional imaging techniques including computed tomography/magnetic resonance perfusion, texture analysis, diffusion-weighted imaging, and positron emission tomography in terms of prediction of treatment response or prognosis in esophageal cancer.

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## INTRODUCTION

Esophageal cancer is the seventh most common cancer, and sixth leading cause of death in the world<sup>[1]</sup>. Surgical resection is the only curative method for esophageal cancer, but is limited to early stage disease, and the recurrence rate after radical resection of esophageal cancer is reported to be approximately 50%, and most cases of recurrence occur within two years after surgery<sup>[2-4]</sup>.

In this context, personalized or precision medicine, which enables the best choice of treatment based on certain biomarkers, is highly desirable, preventing side-effect and extra expenses, leading to more effective multidisciplinary treatments. Angiogenesis, tumor stroma, hypoxia, heterogeneity, and metabolism are known as typical biological features of malignancies, and these have been investigated to be biomarkers for diagnosis, prognosis, and treatment response. These biological features are usually investigated by the cell and molecular biology, but recent advances in imaging technique enable non-invasive assessment of various tumor functions, which have been investigated their biomarker value in malignancies. Imaging derived markers have the advantage of being non-invasive, spatially resolved and repeatable, compared to bio-specimen biomarkers which are obtained by removing a sample from a patient. Recent increasing interests in “Radiomics”, which is an emerging field that converts imaging data into a high dimensional mineable feature space using a large number of automatically extracted data-characterization algorithms, makes imaging derived biomarkers more valuable. In these contexts, this review will discuss how we can use various imaging derived biomarkers including perfusion analysis using computed tomography (CT) or magnetic resonance imaging (MRI), texture analysis, diffusion-weighted imaging (DWI), and positron emission tomography (PET) in terms of prediction of treatment response or prognosis to improve outcome of esophageal cancer patients.

## PERFUSION ANALYSIS

Perfusion analysis using dynamic contrast-enhanced CT (DCE-CT/CT perfusion) and DCE-MRI (MR perfusion) can quantify tissue hemodynamics by measuring the temporal changes in tissue attenuation after administration of intravenous contrast media<sup>[5-7]</sup>. Since “angiogenesis” plays an important role in almost all types of cancer progression<sup>[8-10]</sup>, quantification of vascular physiology using CT or MR perfusion techniques may reflect tumor angiogenesis, and has a potential to be a biomarker in cancer treatment<sup>[11]</sup>. These perfusion techniques are readily incorporated into the existing CT and MRI protocol, and enables *in vivo* quantification of tissue hemodynamics using the modeling tracer kinetics on the imaging workstation (Figure 1)<sup>[5,6]</sup>. In esophageal cancer, several previous papers reported associations of CT or MR perfusion parameters with treatment response to chemotherapy or chemoradiotherapy (Table 1). In MR perfusion studies, it was reported that higher Ktrans value (a parameter related to vessel permeability and tissue blood flow) before chemoradiation therapy (CRT) was associated with better treatment response<sup>[12,13]</sup>. In CT perfusion studies, Hayano *et al.*<sup>[14]</sup> reported that higher blood flow of the tumor before CRT was associated with better treatment response and overall survival in esophageal squamous cell carcinoma patients. Makari *et al.*<sup>[15]</sup> also reported that high tumor BF measure by CT perfusion might predict good response to neoadjuvant chemotherapy and CRT. Interestingly, all these reports suggested that a higher blood flow/perfusion of the tumor was associated with a better outcome of CRT or chemotherapy. It is reasonable because a higher tumor blood flow/perfusion leads to a better drug delivery and a higher oxygenation, resulting in better chemo- and radio-sensitivity. There are a few paper reporting perfusion change in esophageal cancer during chemotherapy or CRT. Sun *et al.*<sup>[12]</sup> demonstrated that the complete response group showed a significant decrease in Ktrans. Similarly, Hayano *et al.*<sup>[16]</sup> reported that a significant decrease of blood flow in the tumor was observed from CT perfusion study of esophageal cancer, and they reported that patients with a greater reduction

in tumor blood flow during CRT survived significantly longer than those with lower tumor blood flow reduction. It is speculated that the tissue fibrosis due to CRT leads to compression of tumor capillaries and increased flow resistance, results in decrease of blood flow/perfusion after CRT. In fact, it was reported that patients who achieved pathological complete response (pCR) after neoadjuvant CRT had tumors with lower blood flow than non-pCR<sup>[17]</sup>.

Regarding relationship between CT/MR perfusion and angiogenesis, published results are controversial. For example, Chen *et al*<sup>[18]</sup> demonstrated that tumor blood volume measured by CT perfusion was significantly correlated with micro-vessel density in esophageal cancer, while Sato *et al*<sup>[19]</sup> reported that there was no significant correlation of tumor blood flow with the micro-vessel density in CT perfusion study of gastric cancer. Sato *et al*<sup>[19]</sup> speculated that blood flow assessed with perfusion imaging reflected only the functional vessels with a lumen, and not the functionless tumor vascularity; and therefore, micro-vessel density studied immunohistochemically *in vitro* using surgical specimens might be inadequate for “*in vivo*” tumor vascular physiology. These factors may affect controversial results on relationship of CT/MR perfusion with immunohistochemically evaluated angiogenesis.

This perfusion technique using CT and MRI is very interesting and exciting technique with a potential to be a useful biomarker, but this technique is still considered a research tool in the realm of oncology. A consensus and standardization of data acquisition and analysis methods have yet to be established. The definition of the tumor region of interest (ROI) is subject to similar consideration, because the method used to draw the ROI clearly influences the perfusion parameters. Relatively high radiation dose and complicated procedure should be improved to be more common examination in clinical practice of esophageal cancer.

## TEXTURE ANALYSIS

Analysis of texture within tumor on medical imaging such as CT, MRI, and PET, which reflects structural abnormality or heterogeneity in the tumor, is emerging as a potential biomarker to predict prognosis and treatment response in patients with cancer<sup>[20]</sup>, because most malignant tumors show a striking amount of intratumor heterogeneity, which has implications for diagnosis, treatment efficacy, and the identification of drug targets<sup>[21]</sup>. There are various methods including statistical-, model-, and transform-based methods with various texture parameters<sup>[22]</sup>. Common texture parameters are entropy (a measure of irregularity), uniformity (a measure of uniform distribution of grey-levels), skewness (a measure of asymmetry of the histogram), kurtosis (a measure of peakedness and tailedness), and fractal dimension (a measure of complexity)<sup>[23-25]</sup>. Ganeshan *et al*<sup>[26]</sup> reported that tumor heterogeneity (uniformity) assessed on unenhanced CT was correlated with 18F-fluorodeoxyglucose (18F-FDG) uptake, and was an independent predictor of survival in 21 patients with esophageal cancers. Yip *et al*<sup>[27]</sup> reported that post-treatment uniformity and entropy of the tumor measured on contrast-enhanced CT were correlated with overall survival in esophageal cancer patients treated with CRT. In fractal analysis of PET imaging, Tochigi *et al*<sup>[28]</sup> reported that the low fractal dimension of tumor 18F-FDG uptake was associated with favorable survival, and they concluded that metabolic heterogeneity measured by fractal analysis can be a novel imaging biomarker for survival in patients with esophageal squamous cell carcinoma.

These texture analysis is a post-processing mathematical technique, which can apply to any medical imaging with no additional radiation exposure, special protocol, and cost, and maximizes the information obtained from current standard medical imaging (Figure 2). This technique still needs further investigation and standardization to be used in clinical practice, but has a potential to be a valuable clinical tool in the management of esophageal cancer.

## DIFFUSION-WEIGHTED IMAGING

In 1905, Einstein described molecular diffusion or Brownian motion formally on the basis of the random translational motion of molecules<sup>[29]</sup>. Recent advances in magnetic resonance gradient technology have allowed acquisition of the apparent diffusion coefficient (ADC) value, which can be calculated by the DWI measurements acquired with a different gradient duration and amplitude (b-values)<sup>[30]</sup>. DWI has been discussed in terms of its biomarker value for cancer treatment in a consensus meeting, and a publication on consensus and recommendations for DWI as a cancer biomarker

**Table 1** Summary of reports on computed tomography or magnetic resonance perfusion in esophageal cancer

	Year	Patients	Biomarker candidate	Prediction of treatment outcome
Hayano <i>et al</i> <sup>[14]</sup>	2007	31	Baseline BF (CT perfusion)	High baseline BF associated with good response and OS after CRT (31)
Makari <i>et al</i> <sup>[15]</sup>	2007	46	Baseline BF (CT perfusion)	High baseline BF associated with good response and OS after chemotherapy (36) and CRT (10)
Djuric-Stefanovic <i>et al</i> <sup>[17]</sup>	2015	40	Post-therapeutic BF (CT perfusion)	Post-therapeutic BF < 30 mL/min/100 g can predict pCR with 100% of sensitivity and specificity
Lei <i>et al</i> <sup>[13]</sup>	2015	25	Baseline Ktrans (MR perfusion)	Ktrans was significantly different between CR and non-CR after CRT
Sun <i>et al</i> <sup>[12]</sup>	2018	59	Change of Ktrans (MR perfusion)	Change in Ktrans was the best parameter to assess treatment response

BF: Blood flow; pCR: Pathological complete response; CRT: Chemoradiation therapy; OS: Overall survival; CT: Computed tomography.

has been published highlighting the potential of this technique in the management of cancer patients (Figure 3)<sup>[51]</sup>.

In esophageal cancer, there are seven papers evaluating DWI for prediction of CRT response and prognosis<sup>[32]</sup>, but the results are controversial (Table 2). In 2011, Aoyagi *et al*<sup>[33]</sup> reported that higher baseline tumor ADC was associated with better survival. Another study also suggested that high baseline tumor ADC was associated with good response to CRT<sup>[34]</sup>, while De Cobelli *et al*<sup>[35]</sup> reported that the high baseline tumor ADC was associated with poor response to CRT. Because of these conflicting results, it is still unclear whether pre-therapeutic tumor ADC can really predict response or survival after CRT. Cheng *et al*<sup>[32]</sup> performed meta-analysis, and reported that change of ADC and post-therapeutic ADC of the tumor were promising reliable and valuable predictor for the response to CRT, rather than pre-therapeutic ADC. Interestingly, Imanishi *et al*<sup>[36]</sup> reported that early increase of tumor ADC (> 15% after 20 Gy) could predict treatment response with 85% of accuracy and 100% of positive predictive value. Similarly, three studies suggested importance of post-CRT ADC and the change of ADC after 2-3 weeks of CRT in terms of prediction of response to CRT in esophageal cancer<sup>[37-39]</sup>.

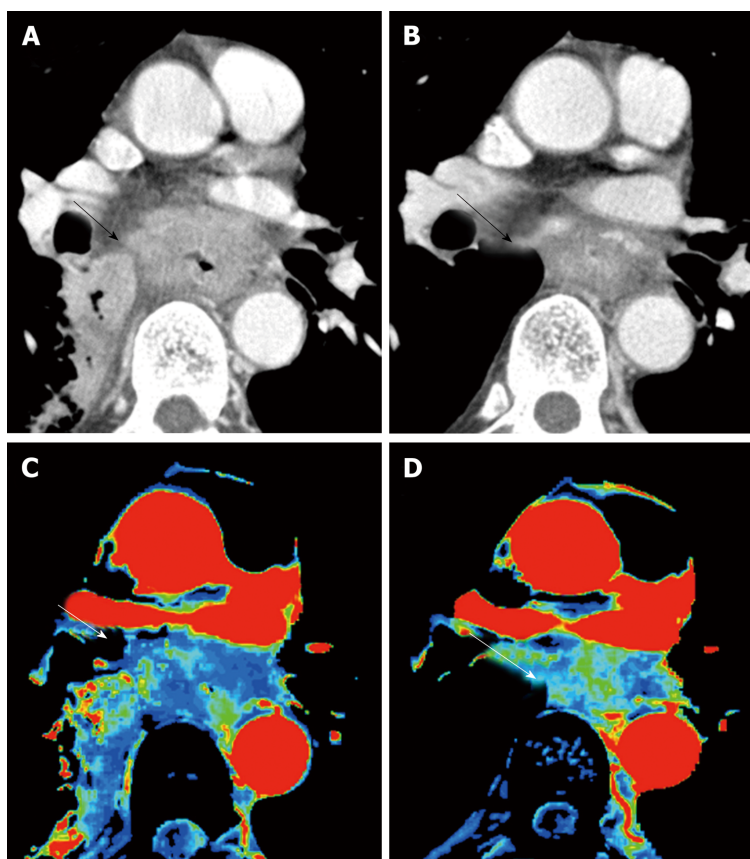
Because DWI does not need radiation exposure and contrast agents, it can be an ideal biomarker. However, standardization of data acquisition and analysis methods have yet to be established for DWI. Low spatial resolution, especially in high b-value image, should be improved for accurate detection and quantification of the tumor.

## POSITRON-EMISSION TOMOGRAPHY

PET is a quantitative imaging technique with use of various types of tracers. 18F-FDG, which can quantify glucose metabolism of the tissue, is the most common clinical PET tracer. Theoretically, malignant tumor cells exhibit strongly enhanced energy consumption, and lead to increased 18F-FDG uptake due to the increased number of glucose transporters and the increased hexokinase activity (Figure 4). The standardized uptake value (SUV) is generally used to quantify the tissue glucose metabolism, which has been reported its biomarker value in the treatment response and prognosis in various types of malignancies.

Regarding the biomarker value of pre-therapeutic PET in surgically treated esophageal cancer, Fukunaga *et al*<sup>[40]</sup> reported that a high SUV of the tumor before surgery had a poorer prognosis compared with those with low FDG uptake in esophageal cancer patients who received curative surgery without neoadjuvant therapy in 1998. After this paper had been published, seven papers on this subject were published<sup>[41-47]</sup>, and all those papers suggested that high tumor SUV before surgery was associated with poor survival in surgically treated esophageal cancer (no neoadjuvant therapy)<sup>[48]</sup>. On the other hand, interestingly, pretherapeutic tumor SUV may not associate with survival in patients who received neoadjuvant chemotherapy





**Figure 1** Perfusion change of esophageal cancer during chemoradiation therapy. A: Computed tomography (CT) image at baseline; B: CT image at post-chemoradiation therapy (CRT); C: Blood flow map by CT perfusion at baseline; D: Blood flow map at post-CRT. Baseline blood flow of this tumor was low, and this patient was diagnosed as no-responder. CRT: Chemoradiation therapy; CT: Computed tomography.

or CRT<sup>[49,50]</sup>, while the tumor SUV after neoadjuvant therapy can be a biomarker for survival. Swisher *et al.* reported that the tumor SUV after CRT is the most accurate test to predict survival in esophageal cancer patients (87% is adenocarcinoma) who were treated CRT followed by curative surgery<sup>[51]</sup>. Higuchi *et al.*<sup>[52]</sup> also demonstrated that post-CRT SUV uptake in the tumor (cut-off 2.5) was the preoperative prognostic factor in esophageal squamous cell carcinoma patients who were treated neoadjuvant CRT or chemotherapy. Regarding change in tumor SUV during neoadjuvant therapy, early decrease (after 2 wk of neoadjuvant therapy) in FDG uptake is reported to be a predictive marker for response and survival<sup>[53-59]</sup>. However, some studies included patients with a wide range of disease (adenocarcinomas and squamous cell carcinomas, stage I through IV), and studies used different neoadjuvant treatment regimens.

Nevertheless, FDG-PET seems to be served as a useful biomarker for treatment response and prognosis in various types of treatments, and we need further investigation with a large multicenter prospective trial to confirm usefulness of FDG-PET in the management of esophageal cancer.

## CONCLUSION

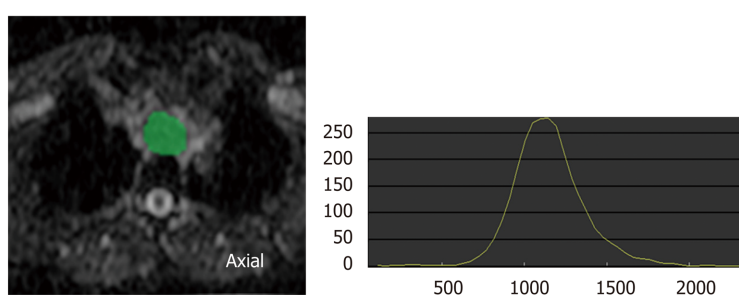
Ideal biomarker should be simple, non-invasive, reproducible, and widely available. Given the wide availability and the less invasiveness, imaging has a big potential to be an ideal biomarker. As we reviewed, various imaging biomarkers showed interesting results, and some of them are ready to use in clinical practice of esophageal cancer patients, which would provide patients more personalized and effective treatment, leading better outcome.



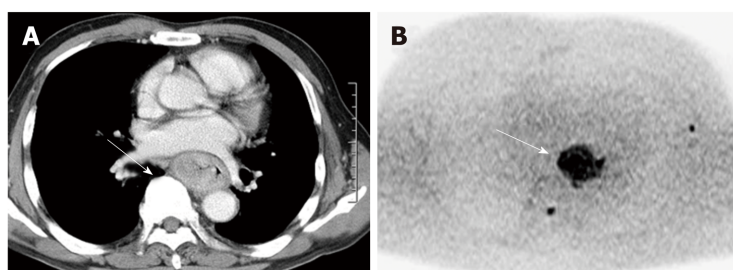
**Table 2** Summary of reports on diffusion-weighted imaging in esophageal cancer

	Year	Patients	Biomarker candidate	Prediction of treatment outcome
Aoyagi <i>et al</i> <sup>[33]</sup>	2011	80	Baseline ADC	High baseline ADC associated with favorable survival after CRT
Imanishi <i>et al</i> <sup>[36]</sup>	2013	27	Early change of ADC, post-CRT ADC	Increase of ADC/high post-CRT ADC associated with good response to CRT
De Cobelli <i>et al</i> <sup>[35]</sup>	2013	32	Baseline ADC	High baseline ADC associated with poor response to CRT
Van Rossum <i>et al</i> <sup>[39]</sup>	2015	20	Early change of ADC	Increase of ADC associated with good response to CRT
Wang <i>et al</i> <sup>[37]</sup>	2016	38	Early change of ADC, post-CRT ADC	Increase of ADC/high post-CRT ADC associated with good response to CRT
Li <i>et al</i> <sup>[38]</sup>	2017	28	Early change of ADC, post-CRT ADC	Increase of ADC/high post-CRT ADC associated with good response to CRT
Cong <i>et al</i> <sup>[34]</sup>	2019	52	Baseline ADC	High baseline ADC associated with good response to RT

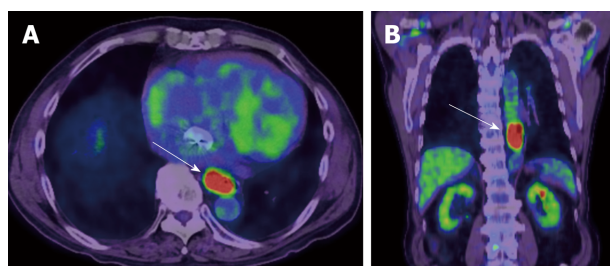
ADC: Apparent diffusion coefficient; CRT: Chemoradiation therapy; RT: Radiation therapy.



**Figure 2** Histogram analysis of diffusion-weighted imaging. Histogram analysis is one of the texture analyses. This is the histogram analysis of apparent diffusion coefficient (ADC) map. Region of interest (ROI) for the tumor is drawn on ADC map, and distribution of pixels in the ROI is quantified as histogram parameters such as kurtosis and skewness. ADC: Apparent diffusion coefficient; ROI: Region of interest.



**Figure 3** Advanced esophageal squamous cell carcinoma in contrast enhanced computed tomography image and diffusion-weighted imaging at  $b = 1000$ . A: Contrast enhanced computed tomography image; B: Diffusion-weighted imaging. The tumor showed conspicuous high signal intensity on high b-value diffusion-weighted imaging.



**Figure 4**  $^{18}\text{F}$ -fluorodeoxyglucose positron emission tomography image of esophageal cancer. A: Axial image; B: Coronal image. Malignancies exhibit strongly enhanced energy consumption, resulting in increased  $^{18}\text{F}$ -fluorodeoxyglucose uptake.

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## Basic Study

# Development and *in vitro* study of a bi-specific magnetic resonance imaging molecular probe for hepatocellular carcinoma

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### Institutional review board

**statement:** This study was approved by the ethics committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

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## Abstract

### BACKGROUND

Hepatocellular carcinoma (HCC) ranks second in terms of cancer mortality worldwide. Molecular magnetic resonance imaging (MRI) targeting HCC biomarkers such as alpha-fetoprotein (AFP) or glypican-3 (GPC3) offers new strategies to enhance specificity and help early diagnosis of HCC. However, the existing iron oxide nanoparticle-based MR molecular probes singly target AFP or GPC3, which may hinder their efficiency to detect heterogeneous micro malignant HCC tumors < 1 cm (MHCC). We hypothesized that the strategy of double antibody-conjugated iron oxide nanoparticles which simultaneously target AFP and GPC3 antigens may potentially be used to overcome the tumor heterogeneity and enhance the detection rate for MRI-based MHCC diagnosis.

### AIM

To synthesize an AFP/GPC3 double antibody-labeled iron oxide MRI molecular probe and to assess its impact on MRI specificity and sensitivity at the cellular level.

### METHODS

A double antigen-targeted MRI probe for MHCC anti-AFP-USPIO-anti-GPC3 (UAG) was developed by simultaneously conjugating AFP and GPC3 antibodies to a 5 nm ultra-small superparamagnetic iron oxide nanoparticle (USPIO). At the



publication of the paper.

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

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same time, the singly labeled probes of anti-AFP-USPIO (UA) and anti-GPC3-USPIO (UG) and non-targeted USPIO (U) were also prepared for comparison. The physical characterization including morphology (transmission electron microscopy), hydrodynamic size, and zeta potential (dynamic light scattering) was conducted for each of the probes. The antigen targeting and MRI ability for these four kinds of USPIO probes were studied in the GPC3-expressing murine hepatoma cell line Hepa1-6/GPC3. First, AFP and GPC3 antigen expression in Hepa1-6/GPC3 cells was confirmed by flow cytometry and immunocytochemistry. Then, the cellular uptake of USPIO probes was investigated by Prussian blue staining assay and *in vitro* MRI (T2-weighted and T2-map) with a 3.0 Tesla clinical MR scanner.

## RESULTS

Our data showed that the double antibody-conjugated probe UAG had the best specificity in targeting Hepa1-6/GPC3 cells expressing AFP and GPC3 antigens compared with single antibody-conjugated and unconjugated USPIO probes. The iron Prussian blue staining and quantitative T2-map MRI analysis showed that, compared with UA, UG, and U, the uptake of double antigen-targeted UAG probe demonstrated a 23.3% (*vs* UA), 15.4% (*vs* UG), and 57.3% (*vs* U) increased Prussian stained cell percentage and a 14.93% (*vs* UA), 9.38% (*vs* UG), and 15.3% (*vs* U) reduction of T2 relaxation time, respectively. Such bi-specific probe might have the potential to overcome tumor heterogeneity. Meanwhile, the coupling of two antibodies did not influence the magnetic performance of USPIO, and the relatively small hydrodynamic size ( $59.60 \pm 1.87$  nm) of double antibody-conjugated USPIO probe makes it a viable candidate for use in MHCC MRI *in vivo*, as they are slowly phagocytosed by macrophages.

## CONCLUSION

The bi-specific probe presents enhanced targeting efficiency and MRI sensitivity to HCC cells than singly- or non-targeted USPIO, paving the way for *in vivo* translation to further evaluate its clinical potential.

**Key words:** Hepatocellular carcinoma; Molecular imaging; Magnetic resonance imaging; Ultra-small superparamagnetic iron nanoparticles; Alpha-fetoprotein; Glypican-3

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**Core tip:** The single targeting of existing hepatocellular carcinoma-targeted magnetic resonance imaging (HCC-targeted MRI) probes may weaken the detection efficiency due to biomarker associated tumor heterogeneity. Here, double antibody-conjugated ultra-small superparamagnetic iron nanoparticles (USPIO) were synthesized to simultaneously target HCC markers of alpha-fetoprotein (AFP) and glypican-3 (GPC3) antigens in Hepa1-6/GPC3 cells. Such probe showed higher cancer cell labeling efficiency than singly- or non-targeted USPIO probes by Prussian blue staining and *in vitro* MRI, indicating enhanced specificity and sensitivity of MRI diagnosis for micro hepatocellular carcinoma (MHCC). Meanwhile, USPIO with a small core (~5 nm) and hydrodynamic size (~60 nm) after antibody labelling may undergo slow phagocytosis, which could enhance liver tumor MRI contrast in the animal or clinical trial study.

**Citation:** Ma XH, Wang S, Liu SY, Chen K, Wu ZY, Li DF, Mi YT, Hu LB, Chen ZW, Zhao XM. Development and *in vitro* study of a bi-specific magnetic resonance imaging molecular probe for hepatocellular carcinoma. *World J Gastroenterol* 2019; 25(24): 3030-3043

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the major type of primary malignant liver tumor, and it has a high incidence rate and ranks second in terms of cancer mortality worldwide<sup>[1,2]</sup>. Surgical resection is one of the most effective methods for treating

HCC. However, only 10%–15% of the patients can be operated on when diagnosed, because most HCC patients present with a locally advanced stage disease or distant metastasis. It is encouraging that for micro hepatocellular carcinoma (MHCC) patients with tumors smaller than 1 cm in diameter and without lymph node metastasis and local invasion, the 5-year survival rate after radical operation can reach >70%<sup>[3,4]</sup>. Therefore, early and timely diagnosis of MHCC could help improve the success of surgery and significantly improve patients' survival rates.

Non-invasive imaging is the most convenient and effective way to diagnose MHCC in patients with no obvious clinical signs. Among the diverse clinical imaging methods, magnetic resonance imaging (MRI) is becoming one of the most important imaging techniques for clinical HCC screening, diagnosis, and therapeutic evaluation. MRI is a comprehensive imaging technique that is used without ionizing radiation and has a potential for quantitative analysis of morphological and functional imaging, based on high resolution of soft tissue and multi-sequence imaging parameters. MRI is sensitive and accurate for diagnosing typical HCCs with tumor diameters larger than 1 cm<sup>[5]</sup>. However, it is still a challenge for MRI to identify benign and malignant hepatic nodules less than 1 cm, mostly due to the low tumor contrast or lack of specificity for MR contrast agents<sup>[6]</sup>.

Recent achievements in targeted molecular MR imaging offer new strategies to enhance specificity and contrast for detecting such small lesions<sup>[7–11]</sup>. One of the most commonly studied HCC-targeted MRI systems utilizes antibody (aptamer)-guided iron oxide nanoparticles as probes, which are intended to bind specifically with unique overexpressed HCC-related antigens or genes, such as alpha-fetoprotein (AFP) or glypican-3 (GPC3)<sup>[12–15]</sup>. AFP is a clinically widely used HCC serum bio-marker that is secreted from the cytoplasm. The specificity and sensitivity of AFP are 76%–96% and 40%–65%, respectively, whereas the false-positive and false-negative detection rates are approximately 40% and are easily affected by other liver diseases or tumors<sup>[16–18]</sup>. GPC3 is a heparan sulfate proteoglycan linked to the cell membrane by glycosylphosphatidylinositol. It is involved in regulating HCC cell proliferation and potentially serves as an HCC tissue biomarker<sup>[19,20]</sup>. GPC3 expression is highly specific to HCC tumors (84.6%), and its mRNA expression level is even higher than that of AFP, especially for tumors smaller than 3 cm<sup>[19,21]</sup>.

However, the drawback of most existing HCC-targeted MRI molecular probes is that the singularity of the target may weaken the detection specificity and sensitivity, considering the tumor heterogeneity and false positive or false negative diagnoses associated with cancer biomarkers. Therefore, a more complex targeted probe design such as double antigen-targeted probes are well appreciated to precisely capture the molecular features of tumors<sup>[22,23]</sup> and, hence, are expected to further enhance the precision and imaging quality of small HCC or MHCC lesions.

Therefore, we developed a double antigen-targeted MRI probe for MHCC by simultaneously conjugating AFP and GPC3 antibodies to a 5 nm ultra-small superparamagnetic iron oxide nanoparticle (USPIO). USPIOs with a small core size (5 nm) were chosen because their slow phagocytosis by macrophages could make them ideal for liver tumor MRI in future *in vivo* studies or clinical trials<sup>[24–26]</sup>. The aim of the current research was to explore the feasibility of using a doubly targeted HCC MRI molecular probe for cancer labeling at the cellular level. A bi-specific USPIO probe, as well as single-targeting probes conjugated with only AFP or GPC3 antibodies and unlabeled USPIO, was prepared and studied in the murine hepatoma cell line Hepa1-6/GPC3, in terms of their selectivity towards AFP and GPC3 antigens and their T2 MRI properties *in vitro* on a 3.0 Tesla clinical scanner.

## MATERIALS AND METHODS

### Materials

N-succinimidyl ester-functionalized 5 nm USPIOs were from Sigma-Aldrich (catalog #747440, Saint Louis, MO, United States). AFP antibodies were purchased from Abcam Company (ab213328, Cambridge, United Kingdom) and R&D Systems, Inc. (MAB1368, Minneapolis, United States). GPC3 antibodies were obtained from Abcam (ab66596) and R&D Systems, Inc. (MAB2119, Minneapolis, United States). Other chemical reagents were from Sigma-Aldrich and were of analytical grade.

### Preparation of antibody-conjugated USPIO probes

The 5 nm USPIO (abbreviated as U) was N-succinimidyl ester-functionalized, which enabled its efficient conjugation with primary amines of antibodies by amide bond formation between them. Single and double antibody-conjugated USPIOs were synthesized separately as anti-AFP-USPIO (UA), anti-GPC3-USPIO (UG), and anti-

AFP-USPIO-anti-GPC3 (UAG). For single antibody-conjugated probes, 18 mg/mL USPIO was reacted separately with AFP and GPC3 antibodies (400 µg/mL) in 1 mL phosphate-buffered solution (PBS, pH 7.4). For double antibody-conjugated probe, 18 mg/mL USPIO was reacted with equal amounts of AFP and GPC3 antibodies (400 µg/mL each) in a final volume of 1 mL. The mixture was gently stirred and allowed to react for 3 hours at room temperature. Each product was then purified by ultrafiltration with 1 × PBS (pH 7.4) for three cycles using 100 kDa MWCO centrifugal filter (Amicon Ultra-0.5) to remove the uncoupled antibodies. The probes were stored at 4 °C for future experiments.

### Physical characterization of USPIO probes

The morphology, average size, and size distribution of USPIO probes were characterized by transmission electron microscopy (TEM; FEI Tecnai G2 F30, United States) at an acceleration voltage of 300 kV. TEM samples were prepared by dropping each probe solution onto a 400-mesh copper grid with carbon film. The hydrodynamic diameters and zeta potential of the U, UA, UG, and UAG probes were measured by dynamic light scattering (DLS; Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, United Kingdom). Each sample was diluted with double-distilled water and measured in the non-invasive back scatter (NIBS) mode at 25 °C with a scattering angle of 173°.

To deduce transversal relaxivity  $r_2$  of USPIO, the transversal relaxation time  $T_2$  of USPIO water solution at different iron concentrations (0.25, 0.5, 0.75, 1, 1.5, and 2 mmol/L) were measured using a 3.0 Tesla clinical MR scanner (750W, GE Healthcare, United States) with 8-channel head coil.  $T_2$  images were acquired using spin echo (SE) sequence with different TE ranging from 10 ms to 170 ms. The parameters were set as follows: TR = 2000 ms, TE = 10, 20, 30, 40, 50, 70, 80, 90, 110, 130, 150, or 170 ms, matrix = 256 × 256, field of view (FOV) = 20 mm × 20 mm, and slice thickness/slice separation = 3 mm/3.3 mm, and NEX = 2.0.

### Cell culture

The murine hepatoma cell line Hepa1-6 was purchased from ATCC (CRL-1830; Manassas, VA, United States). GPC3-expressing Hepa1-6 cell line (Hepa1-6/GPC3) was developed according to the established protocol<sup>[27]</sup> and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1 mg/mL G418 (Invitrogen, CA, United States) at 37 °C in a 5% CO<sub>2</sub> atmosphere.

### Cell experiment procedure

Cellular experiments were performed as illustrated schematically in Figure 1. First, AFP and GPC3 antigen expression in Hepa1-6/GPC3 cells was confirmed by flow cytometry and immunocytochemistry. Then, the cellular uptake of USPIO probes was investigated by Prussian blue staining assay and *in vitro* MRI. The detailed experimental and analysis methods are described below.

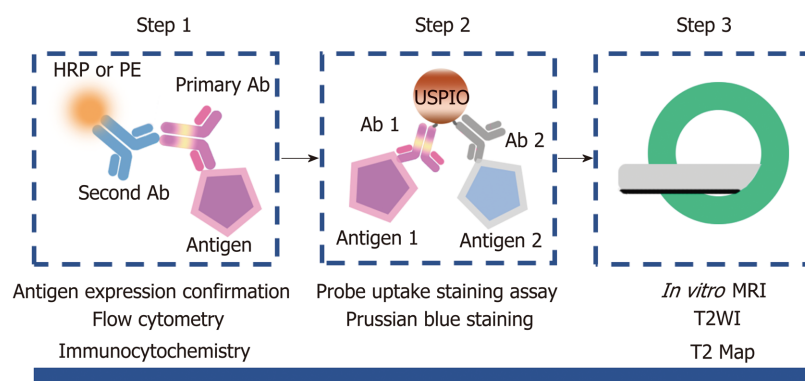
### Confirmation of antigen expression

Expression of AFP and GPC3 antigens in Hepa1-6/GPC3 cells was confirmed by flow cytometry and immunocytochemistry, based on indirect fluorescence/chemical horseradish peroxidase (HRP) labeling.

**Flow cytometry analysis:** The rabbit anti-mouse AFP (Abcam, ab213328) and rabbit anti-mouse GPC3 (Abcam, ab66596) antibodies were used as primary antibodies, respectively, which were further binding with the secondary antibody of PE-conjugated F(ab')<sub>2</sub>-donkey anti-rabbit IgG (12-4739-81, eBioscience) for flow cytometry measurement. For intracellular staining for AFP, cells first experienced fixation and permeabilization, followed by staining with rabbit anti-mouse AFP (Abcam, ab213328) and PE-conjugated F(ab')<sub>2</sub>-donkey anti-rabbit IgG (Cat#: 12-4739-81, eBioscience). Data were acquired using an LSR-II instrument (BD, CA, United States) and analyzed using FlowJo software (Tree Star, OR, United States).

**Immunocytochemistry:** Hepa1-6/GPC3 cells were seeded in an 8-well chamber slide (ThermoFisher) at a density of  $2 \times 10^4$  cells per well and allowed to attach the coverslips for 24 hours. Following incubating cells with 1 µg/ml rabbit anti-mouse AFP monoclonal antibody (Abcam, ab213328) or rabbit anti-mouse GPC3 polyclonal antibody (Abcam, ab66596), cells were processed with the Horseradish Peroxidase (HRP) Color Development Kit (PV-9001, ZSGB-BIO, China). Cells blocked with 10% goat serum were used as a control. 3,3'-diaminobenzidine (DAB) staining was subsequently performed and hematoxylin staining was finally processed for blue cell nuclei. The 8-well chamber was then ready for bright-field optical microscopy.

### Uptake of USPIO molecular probes by Hepa1-6/GPC3 cells



**Figure 1 Flow of cell-based experiments.** First, AFP and GPC3 antigen expression on Hepa1-6/GPC3 cells was confirmed by flow cytometry and immunocytochemistry (step 1). Next, the cellular uptake of USPIO probes was investigated by performing Prussian blue-staining assays for iron (step 2). Finally, *in vitro* MRI was performed, including T2-weighted imaging (T2WI) and T2 Map imaging (step 3). HRP: Horseradish peroxidase; PE: Phycoerythrin; Ab: Antibody; USPIO: Ultra-small superparamagnetic iron oxide; MRI: Magnetic resonance imaging; T2WI: T2-weighted imaging.

**Prussian blue staining assay:** Prussian blue staining was utilized to visibly assess the probes' targeting efficiency and the corresponding iron uptake by cells that were treated with four different molecular probes including U, UA, UG, and UAG. Hepa1-6/GPC3 cells were seeded in an 8-well chamber slide (ThermoFisher) at a density of  $2 \times 10^4$  cells in each chamber and incubated for 4 h with one molecular probe at a concentration of  $50 \mu\text{g Fe/mL}$ . The cells were gently rinsed three times with  $1 \times \text{PBS}$  and fixed with 4% paraformaldehyde for 30 min at RT. After washing three more times with  $1 \times \text{PBS}$ , the resulting cells were incubated with Prussian blue staining solution (Prussian Blue Staining Kit, Solarbio, China) for 15 min, washed with ultrapure water, and were then ready for microscopic observation. The cells that appeared blue were counted to determine the percentage of all cells that efficiently internalized iron or the probe. Each cell-adhering chamber was divided into a  $3 \times 3$  matrix for stained cell counting.

***In vitro* MRI:** Cells were seeded in six-well plates in 2 mL culture medium at a density of  $1 \times 10^6$  cells/well and incubated for 24 h. Four kinds of probes (U, UA, UG, and UAG) were dissolved in fresh cell culture medium and incubated in each well with attached cells and  $100 \mu\text{g Fe/mL}$  for 4 h ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ). Blank cell samples were also prepared by substituting the same volume of  $1 \times \text{PBS}$  with nanoprobe. After the incubation, the cells were washed three times with  $1 \times \text{PBS}$  and detached using  $150 \mu\text{L}$  trypsin per well. After centrifugation, the cells were suspended in  $300 \mu\text{L}$  of 1% agarose gel in PBS and quickly transferred to a 96-well plate and were ready for MRI scanning after concretion at RT.

*In vitro* MRI was performed using a 3.0 Tesla clinical MR scanner (750W, GE Healthcare, United States) with an 8-channel head coil. T2 images were acquired using spin echo (SE) sequence with different multi-echo TE time ranging from 10 ms to 170 ms. The parameters were set as follows: TR = 2000 ms; TE = 10, 20, 30, 40, 50, 70, 90, 110, 130, 150, or 170 ms; matrix =  $256 \times 256$ ; FOV =  $20 \text{ mm} \times 20 \text{ mm}$ ; and slice thickness/slice separation =  $3 \text{ mm}/3.3 \text{ mm}$ ; NEX = 2.0. The T2 values for each sample were fitted as an exponential decay constant from signal intensity *vs* multi-echo TE time curves.

### Statistical analysis

Quantitative data are described as the mean  $\pm$  SD. The Kolmogorov-Smirnov test was used to evaluate whether the continuous variables are normally distributed. Differently treated cell groups were statistically compared using the Mann-Whitney *U*-test or Student's *t*-test. Significant differences between two groups was defined as  $P < 0.05$ . The statistical methods of this study were reviewed by Wang SM from National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

## RESULTS



### Physical characterization of USPIO probes

The morphologies of U, UA, UG, and UAG probes were characterized by TEM and are illustrated in Figure 2A-D. The core diameter of the USPIOs was  $\sim 5$  nm ( $4.88 \pm 0.16$  nm), as demonstrated by the TEM images (Figure 2A) and core size-distribution analysis from 147 nanoparticles (Figure 2E). TEM images of UA, UG, and UAG revealed that each probe maintained good dispersion and uniformity in size after antibody conjugation as shown in Figure 2B-D. The T2-weighted MRI contrast enhancement effects of the USPIO in water solutions are shown in Figure 2F (left column). The transversal molar relaxivity  $r_2$  at 3.0 Tesla was extracted as  $42.75 \text{ mM}^{-1} \text{ s}^{-1}$  by linear regression fitting of transversal relaxation rate ( $1/T_2$ ) data *vs* different iron concentrations (Figure 2F, right column). The scheme used to construct the U, UA, UG, and UAG probes is illustrated in Figure 3A, and their hydrodynamic size distributions are shown in Figure 3B (which were determined by analyzing the DLS intensity-distribution data). Table 1 summarizes the hydrodynamic size and zeta potential of the U, UA, UG, and UAG probes. The hydrodynamic size of UA, UG, and UAG were  $56.48 \pm 0.52$  nm,  $54.76 \pm 1.02$  nm, and  $59.60 \pm 1.87$  nm, respectively, which were larger than the unlabeled USPIOs ( $40.46 \pm 0.53$  nm). The larger size was ascribed to conjugation of the antibody to the USPIO surface, which is in accord with TEM results. Following the binding of antibodies, the negative surface charges of UA, UG, and UAG changed to  $-12.74$  mV,  $-11.22$  mV, and  $-10.23$  mV, respectively, compared with  $-26.13$  mV for unlabeled NHS-ester-functionalized USPIO.

### Confirmation of antigen expression

AFP and GPC3 expression in Hepa1-6/GPC3 cells were confirmed by flow cytometry and immunocytochemistry.

The flow cytometry results are presented in Figure 4A-C. The Hepa1-6/GPC3 cells were subjected to intracellular (Figure 4A) and membrane (Figure 4B) staining with a rabbit anti-mouse AFP monoclonal antibody or isotype control of rabbit IgG, followed by incubation with a PE-conjugated anti-rabbit IgG secondary antibody. Staining with the AFP antibody was significantly higher in the cytoplasm (85.4%, mean fluorescence intensity [MFI]: 10.8) than with the cytoplasmic IgG isotype control (47.4%, MFI: 6.5;  $^aP < 0.0001$ ). The AFP antigen was also expressed on the membrane, based on a comparison between with the membrane isotype control and membrane AFP antibodies ( $^bP < 0.01$ ), as shown in Figure 4B. However, AFP was expressed mainly in the cytoplasm, with only minor membrane staining ( $^cP < 0.0001$ ). Membrane staining with a rabbit anti-mouse GPC3 polyclonal antibody in Hepa1-6/GPC3 cells showed greater fluorescence (71.6% positive, MFI: 11.1) than the isotype control of rabbit IgG (46.8% positive, MFI: 5.92;  $^dP < 0.01$ ). GPC3 was clearly expressed on the cell membrane, although the expression level was not very high. HRP-based immunological staining showed similar staining patterns for AFP and GPC3 in Hepa1-6/GPC3 cells, in which yellow-brown staining appeared (Figure 5), in contrast to the isotype control.

### Uptake of USPIO molecular probes by Hepa1-6/GPC3 cells

The *in vitro* uptake of four kinds of USPIOs was investigated by Prussian blue staining and cellular MRI.

**Prussian blue staining assay:** Figure 6A-E shows Prussian blue-staining images of control-treated Hepa1-6/GPC3 cells and cells treated with unlabeled U, UA, UG, and UAG probes, respectively. The table in Figure 6F summarizes the percentages of stained cells treated with different kinds of USPIO probes. Cells incubated with the UAG probe possessed the highest staining percentage ( $\sim 90\%$ ,  $n = 119$ ) compared with the other three kinds of probes. The staining percentage in the UAG-treated cell group increased 23.3% (*vs* UA,  $n = 133$ ), 15.4% (*vs* UG,  $n = 199$ ), and 57.3% (*vs* U,  $n = 84$ ) compared with UA-, UG-, and U-treated groups, respectively. Meanwhile, the single antibody-conjugated USPIOs also had higher cell binding efficiency relative to the unlabeled USPIO. The higher-level staining results revealed specific binding of antibody-labeled probes to the cellular antigens, AFP and GPC3.

***In vitro* MRI results:** The double antibody-conjugated USPIO probe was designed for MRI of MHCC, with the aim of enhancing the detection specificity and sensitivity. To evaluate the targeting specificity and imaging capacity of such functionalized probe, *in vitro* MRI measurements were performed for Hepa1-6/GPC3 cell samples treated with USPIO probes. Figure 7A and B illustrates the T2WI and T2 map of Hepa1-6/GPC3 cells incubated with unlabeled USPIO, UA, UG, or UAG probes at  $100 \mu\text{g Fe/mL}$ . The mean intensity for these four kinds of cell samples *vs* the corresponding TE values was plotted to calculate T2 values by exponential fitting (Figure 7C). The derived T2 values of 154.83 ms (blank control), 118.31 ms (U), 117.2 ms (UA), 110.02



**Table 1 Hydrodynamic size and zeta potential of different ultra-small superparamagnetic iron oxide probes**

USPIO probe	Hydrodynamic size (nm)	Zeta potential (mV)
USPIO	40.46 ± 0.53	-26.13
Anti-AFP-USPIO	56.48 ± 0.52	-12.74
Anti-GPC3-USPIO	54.76 ± 1.02	-11.22
Anti-AFP-USPIO-anti-GPC3	59.60 ± 1.87	-10.23

USPIO: Ultra-small superparamagnetic iron oxide.

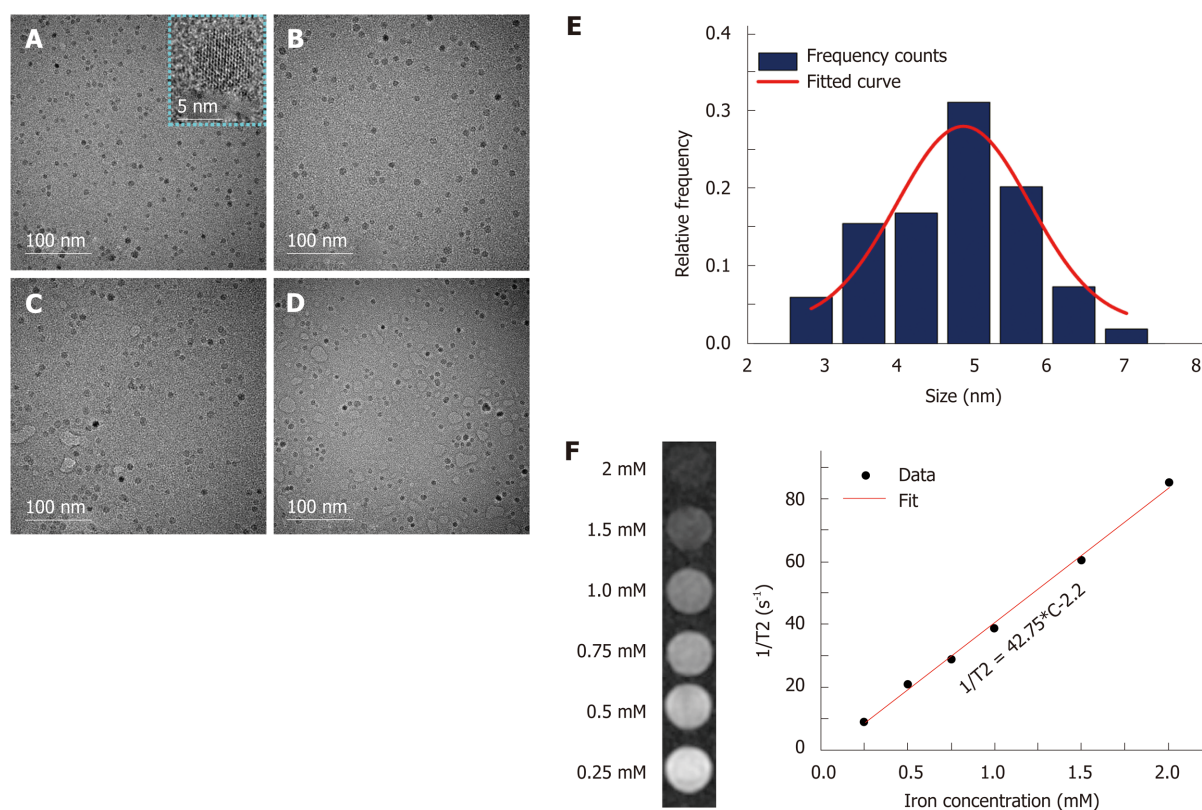
ms (UG), and 99.7 ms (UAG) are summarized in the inset table of **Figure 7C**. The largest reduction of the T2 value was observed with UAG-treated cell samples, which showed a 14.93%, 9.38%, and 15.3% reduction compared with UA, UG, and unlabeled USPIO, respectively. According to the darkest T2-weighted image of UAG-treated cells in **Figure 7A**, the results indicated that the largest amounts of iron or USPIO were bound to or internalized in Hepa1-6/GPC3 cells *via* the targeted antigens. In addition, comparing the T2 imaging results between double and single antibody-conjugated probes suggested that the double antibody-labeled USPIO probe showed enhanced binding efficiency.

## DISCUSSION

The performance of double antibody-conjugated USPIO binding to cells was studied to examine the antigen-targeting ability and the potential as MRI probes for HCC.

Based on simultaneous expression of AFP and GPC3 in Hepa1-6/GPC3 cells, it was clearly demonstrated (both by Prussian blue staining and MRI) that the targeting efficiency of the double antibody-conjugated USPIO probe was higher than that of the single antibody-conjugated probes and unlabeled USPIO. Flow cytometry demonstrated that AFP and GPC3 were expressed mainly in the cytoplasm and membrane, respectively. Referring to other cellular studies that suggested a safe USPIO dosing range of  $\leq 100 \mu\text{g Fe/mL}$ <sup>[28,29]</sup>, a moderate probe concentration of  $50 \mu\text{g Fe/mL}$  and a 4-hour incubation time were chosen for Prussian blue iron staining in this study. While considering the MRI signal sensitivity, a higher probe dosage of  $100 \mu\text{g Fe/mL}$  was used for the *in vitro* cellular MRI experiments. In these experimental situations, the iron-internalization difference between UAG, UA, UG, and unlabeled USPIO probes could still be distinguished by statistical analysis of the percentage of Prussian blue-stained cells and the reduction of T2 values from *in vitro* MRI. The *in vitro* MRI results showed that the UAG probe-treated cells had the most significant reduction in the T2 value, followed by the UG group that possessed a smaller T2 value than the UA group, all of which were smaller than the T2 values of the unlabeled USPIO-treated group and the blank control group. At a lower probe dosage of  $50 \mu\text{g Fe/mL}$ , the Prussian blue-staining results suggested a similar variation tendency, in which the UAG-treated group demonstrated the highest percentage of blue-stained cells among all of the comparison groups. Thus, three points can be discerned. First, the cellular-targeting effect of USPIO probes occurred through a combination of AFP and GPC3 antibodies and the corresponding antigens. Second, the MRI sensitivity of the USPIO probes was related to the expression level of the targeted antigens, and double biomarker-labeled probes may have the potential to overcome the tumor heterogeneity and enhance the imaging sensitivity. Third, the antibody binding did not significantly influence the magnetic properties of USPIO during MRI.

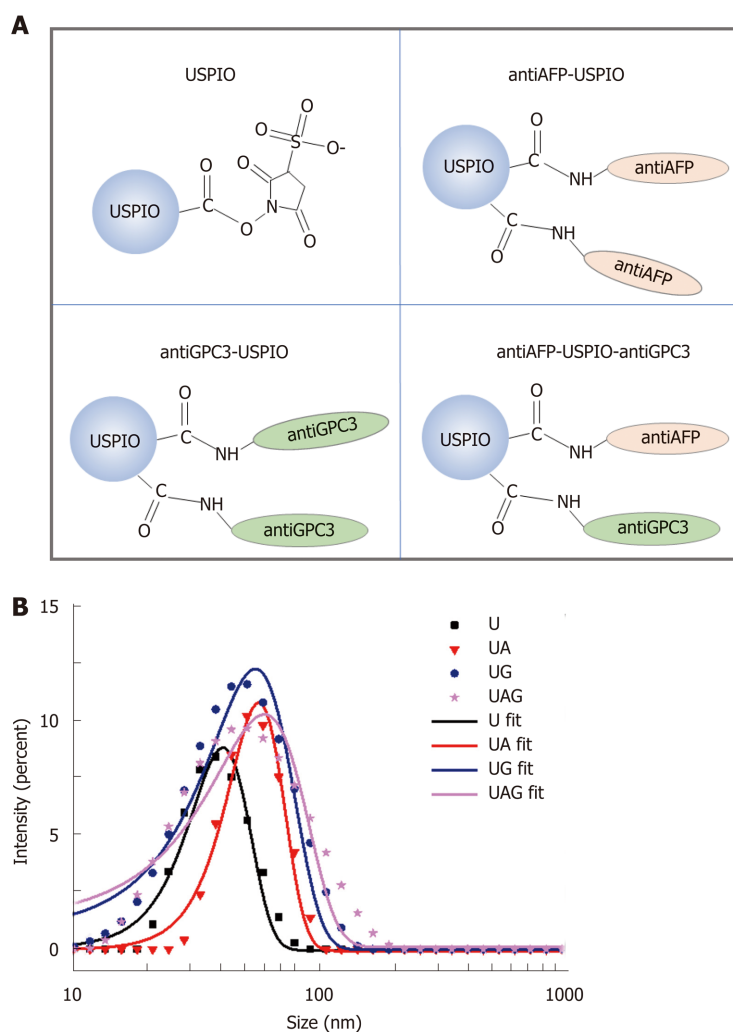
Considering the effects of vascular permeability on most solid tumors and phagocytosis by the mononuclear phagocytic system, the probe's hydrodynamic size plays an important role in entering the tumor<sup>[25,26,30]</sup>. In our study, the USPIO with a small core size ( $\sim 5 \text{ nm}$ ) was adopted as the platform to further conjugate with targeting biomarkers such as AFP and GPC3 antibodies. The hydrodynamic sizes of the probes ranged from 40 nm to 59.6 nm after single or double antibody conjugation to the USPIO particles. Such size range was appropriate for *in vivo* studies, as it may help avoid leakage into the blood or fast clearance and phagocytosis by macrophages rich in normal liver tissue, which could facilitate specific probe binding to tumor antigens with a low level of background signal and clearance by the immune system<sup>[24,31-33]</sup>.



**Figure 2** Physical characterization of ultra-small superparamagnetic iron oxide probes by transmission electron microscopy and magnetic resonance imaging. A-D: Transmission electron microscopy (TEM) images of ultra-small superparamagnetic iron oxide (USPIO) probes of U, UA, UG, and UAG, respectively. E: The core size distribution of USPIO with a mean diameter of 4.88 nm and a standard deviation of 0.16 nm ( $n = 147$ ), as determined from the TEM images. F: T2-weighted magnetic resonance images of a series of water solutions containing different concentrations of USPIO as indicated by iron concentration (left) and linear regression fitting of the transversal relaxation rate ( $1/T_2$ ) data vs different iron concentrations for extracting the transverse relaxivity  $r_2$  (right). UAG: Anti-AFP-USPIO-anti-GPC3; UA: Anti-AFP-USPIO; UG: Anti-GPC3-USPIO; U: Unlabeled (non-targeted) USPIO.

The present study had several limitations. First, an NHS-ester-functionalized USPIO was chosen as the basic nanoplatform for covalent conjugation with amino groups on the antibodies. Such random conjugation may block some antibody-binding sites and decrease the binding efficiency of the probes. Second, because the AFP and GPC3 antibodies had similar molecular weights (~65 kDa), the only quantification control during probe synthesis was to add the same quantity of each antibody. The exact number of labeled antibodies was not quantified, and we lacked a reference for controlling the precise ratio of the different antibodies. Third, the iron content in the study was just enough to present differences in MRI signal changes between each USPIO probe. A noticeable difference may require a further increase in the iron concentration, especially for *in vivo* experiments.

Several issues require further study in the future. In this study, the HCC biomarkers, AFP and GPC3, were chosen based on clinical considerations. The cytoplasmic expression of AFP raised the complexity of the study in terms of probe internalization. The binding of UA with AFP antigens could be inferred by comparing the Prussian blue-stained cell percentage and T2 reduction between the UA- and unlabeled USPIO-treated samples, although the differences were not significant. We hypothesized that AFP proteins secreted into the membrane play a main role in USPIO binding-induced reduction of the T2 relaxation time during *in vitro* MRI. However, the exact internalization route for such probes and whether the secreted AFP proteins contribute to UA internalization require detailed studies in the future. In addition, monoclonal antibodies against AFP and GPC3 were chosen in the study to ensure the specificity and purity. In future *in vivo* studies or investigation of cytoplasmic targeting by USPIO, small antibody fragments possessing even smaller molecular weights might generate improved results. The shrinkage of the hydrodynamic size may induce elongation of blood circulation time and shorter period of time reaching the best tumor-to-background contrast. Furthermore, the surface coating is an equally important factor for *in vivo* fate of nanoparticle-based probes. Compared with hydrophobic coatings, hydrophilic surface may help nanoparticles to avoid plasma protein adsorption and accumulation, which could lead

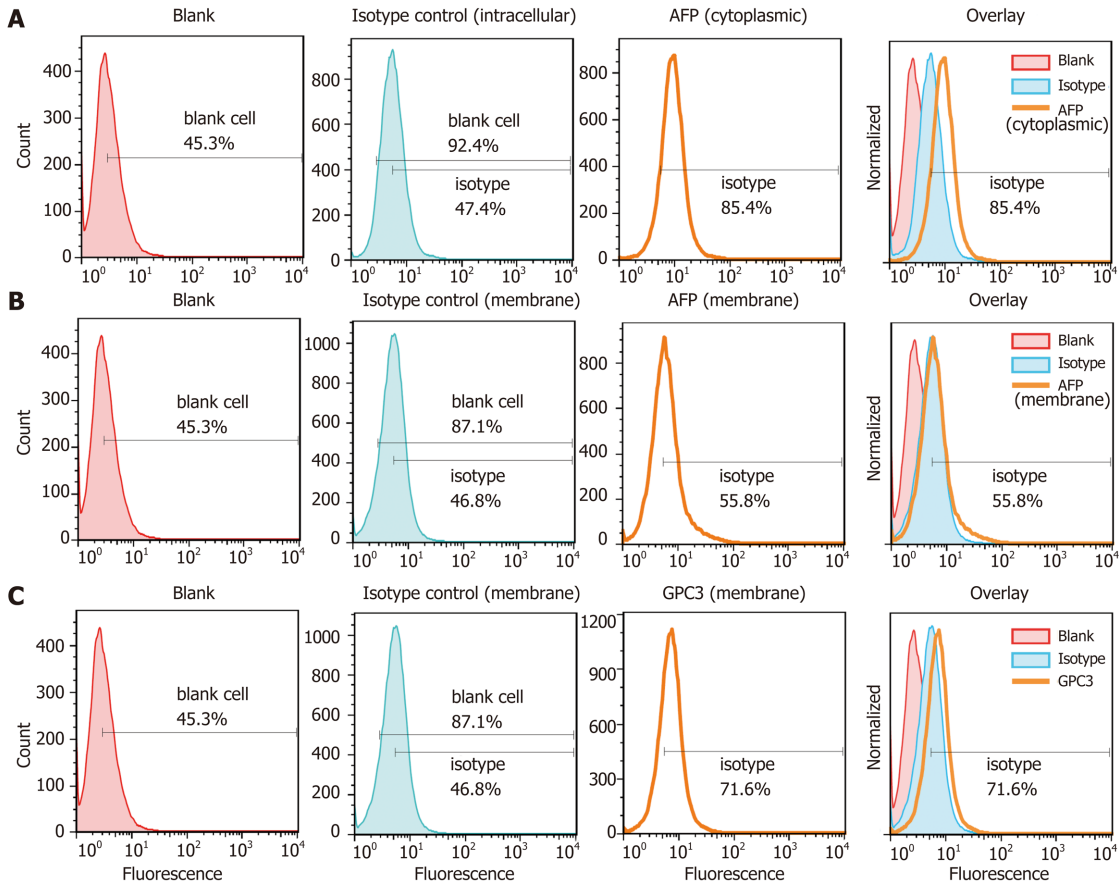


**Figure 3 Hydrodynamic size distribution of antibody-conjugated ultra-small superparamagnetic iron oxides.**

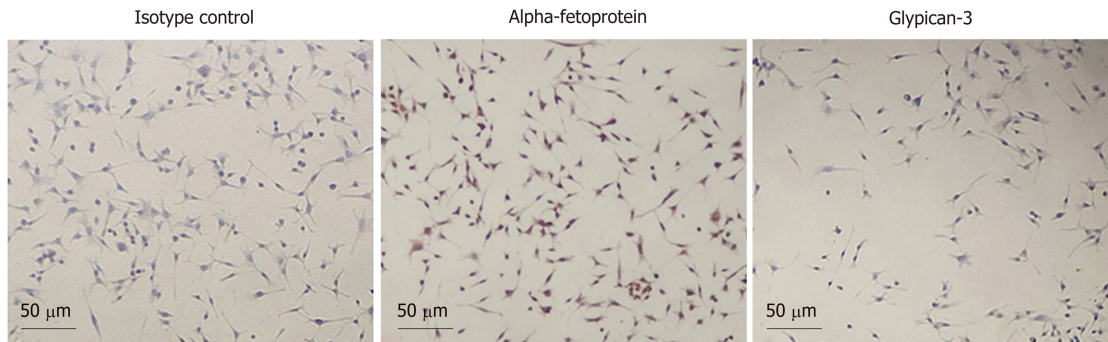
A: Schematic illustration of the conjugation between antibodies (anti-AFP and anti-GPC3) and USPIO-NHS ester to form single or double antibody-conjugated USPIO probes. B: Hydrodynamic size distribution of USPIO (U), anti-AFP-USPIO (UA), anti-GPC3-USPIO (UG), and anti-AFP-USPIO-anti-GPC3 (UAG). USPIO: Ultra-small superparamagnetic iron oxide; AFP: Alpha-fetoprotein; GPC3: Glypican-3; NHS ester: Succinimidyl ester.

to reticuloendothelial system (RES) or mononuclear phagocytic system recognition and uptake<sup>[34]</sup>. Therefore, to further reduce non-specific uptake of USPIO by the RES system, surface modifications, such as hydrophilic PEG coatings for the USPIO, could be also considered in the *in vivo* experiments.

In conclusion, USPIO conjugated with antibodies against two biomarkers (AFP and GPC3) were synthesized as an HCC MRI probe and evaluated using a murine hepatoma cell line expressing GPC3. The coupling of multiple antibodies did not weaken or influence the magnetic performance of USPIO, and the double antibody-conjugated USPIO probe targeted the cancer cells with higher efficiency and sensitivity than single antibody-labeled USPIO probes. Therefore, the multi-targeting strategy may be potentially applied in MRI probe design to overcome the tumor heterogeneity and enhance sensitivity for animal experiments and early clinical diagnosis of MHCC. The current study contributes preliminary data to support future *in vivo* or clinical investigations. The further validation or optimization of the probe to enhance the circulation time and suppress the background signal from normal liver, including the hydrodynamic size and surface coatings, is expected in the future *in vivo* experiments.

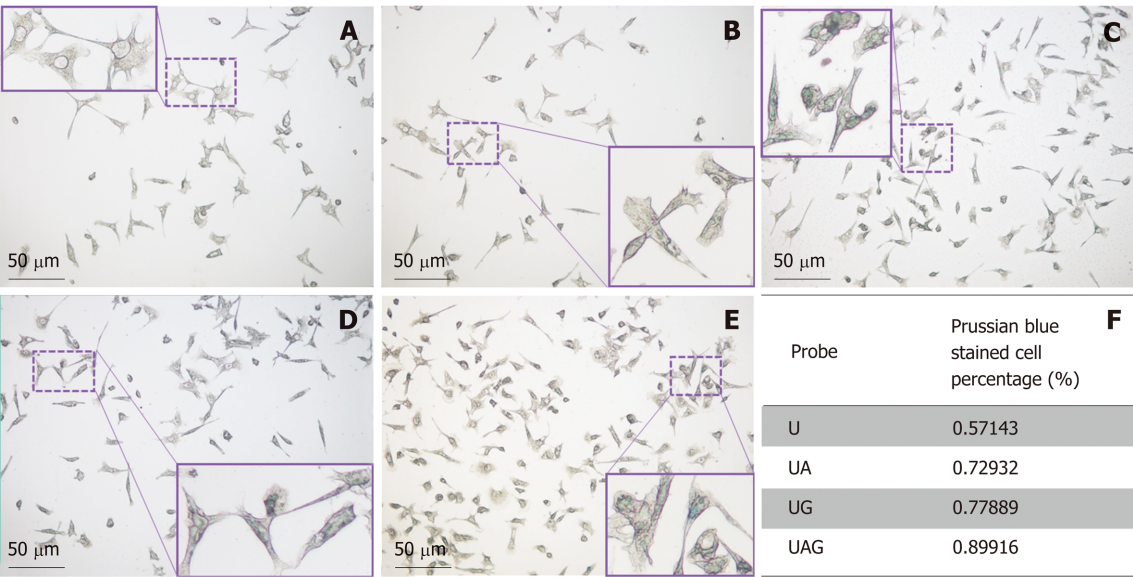


**Figure 4** Detection of alpha-fetoprotein and glypican-3 antigen expression in Hepa1-6/GPC3 cells by flow cytometry. Flow cytometry data showed significantly higher alpha-fetoprotein expression in the cytoplasm (A) than in the membrane (B), compared with blank cell and IgG isotype controls. C: The positive shift of fluorescence distribution compared with isotype control illustrated higher membrane expression of the glypican-3 antigen. AFP: Alpha-fetoprotein; GPC3: Glypican-3.

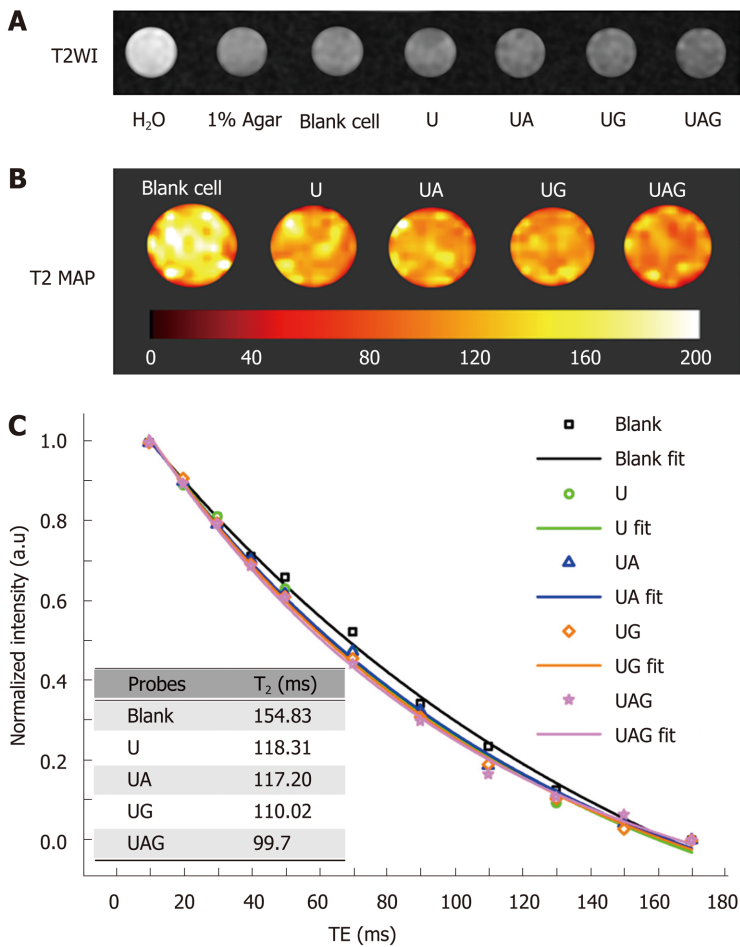


**Figure 5** Cellular immunocytochemistry results. From left to right: Horseradish peroxidase-based immunological staining with IgG isotype control, anti-alpha-fetoprotein antibody, and anti-glypican-3 antibody.





**Figure 6** Prussian blue staining of Hepa1-6/GPC3 cells treated with four kinds of ultra-small superparamagnetic iron oxide probes. Prussian blue-staining images of blank Hepa1-6/GPC3 cells (A) and Hepa1-6/GPC3 cells treated with 50  $\mu\text{g}$  Fe/mL of (B) USPIO (U), (C) anti-AFP-USPIO (UA), (D) anti-GPC3-USPIO (UG), or (E) anti-AFP-USPIO-anti-GPC3 (UAG). (F) Quantitation of the percentages of blue stained cells. The total counted cell number for the U, UA, UG, and UAG groups was 84, 133, 199, and 119, respectively. USPIO: Ultra-small superparamagnetic iron oxide; AFP: Alpha-fetoprotein; GPC3: Glypican-3; UAG: Anti-AFP-USPIO-anti-GPC3; UA: Anti-AFP-USPIO; UG: Anti-GPC3-USPIO; U: Unlabeled (non-targeted) USPIO.



**Figure 7** *In vitro* magnetic resonance imaging results demonstrating the binding efficiency and imaging properties of different ultra-small superparamagnetic iron oxide probes. A: T2WI of different samples contained in a 96-well plate. From left to right:  $\text{H}_2\text{O}$ , 1% agar, blank Hepa1-6/GPC3 cells, and hepa1-6/GPC3 cells treated with 100  $\mu\text{g}$  Fe/mL USPIO (U), anti-AFP-USPIO (UA), anti-GPC3-USPIO (anti-GPC3-USPIO), or anti-AFP-USPIO-anti-GPC3 (UAG). B: Pseudocolor T2 map of cell samples treated with U, UA, UG, and UAG, respectively, compared with blank cells. T2 values are illustrated with a color bar. C: Signal



intensities of cells after different probe treatments under different TE and exponential fits for the T2 values. Inset: The fitted T2 relaxation time for cells treated with different USPIO probes (blank control, U, UA, UG, or UAG). USPIO: Ultra-small superparamagnetic iron oxide; AFP: Alpha-fetoprotein; GPC3: Glypican-3; UAG: Anti-AFP-USPIO-anti-GPC3; UA: Anti-AFP-USPIO; UG: Anti-GPC3-USPIO; U: Unlabeled (non-targeted) USPIO; T2WI: T2-weighted imaging.

## ARTICLE HIGHLIGHTS

### Research background

Hepatocellular carcinoma (HCC) ranks second in terms of cancer mortality worldwide. Molecular magnetic resonance imaging (MRI) targeting HCC biomarkers such as alpha-fetoprotein (AFP) or glypican-3 (GPC3) offers new strategies to enhance specificity and help early diagnosis of HCC. However, the existing iron oxide nanoparticle-based MR molecular probes singly target AFP or GPC3, which may hinder their efficiency to detect heterogeneous micro malignant HCC tumors < 1 cm (MHCC).

### Research motivation

We hypothesized that the strategy of double antibody-labeled iron oxide nanoparticles which simultaneously target AFP and GPC3 antigens may potentially be used to overcome the tumor heterogeneity and enhance detection rate for MRI-based MHCC diagnosis, including the sensitivity and specificity.

### Research objectives

The main objective of the current research was to synthesize an AFP/GPC3-double antibody-labeled iron oxide MR molecular probe and to assess its impact on MRI specificity and sensitivity at the cellular level. The preliminary *in vitro* data could help to optimize the key factors of MRI molecular probe design including labeled biomarkers and hydrodynamic size for future *in vivo* experiments.

### Research methods

The double antigen-targeting MRI probe for MHCC anti-AFP-USPIO-anti-GPC3 (UAG) was developed by simultaneously conjugating alpha-fetoprotein (AFP) and glypican-3 (GPC3) antibodies to a 5 nm ultra-small superparamagnetic iron oxide nanoparticle (USPIO). At the same time, the singly labeled probes of anti-AFP-USPIO (UA), anti-GPC3-USPIO (UG), and non-targeted USPIO (U) were also prepared for comparison. The physical characterization including morphology (transmission electron microscopy), hydrodynamic size, and zeta potential (dynamic light scattering) was conducted for each of the probe. The antigen targeting and MR imaging ability for these four kinds of USPIO probes were studied in the GPC3-expressing murine hepatoma cell line, Hepa1-6/GPC3. First, AFP and GPC3 antigen expression in Hepa1-6/GPC3 cells was confirmed by flow cytometry and immunocytochemistry. Then, the cellular uptake of USPIO probes was investigated by Prussian blue staining assay and *in vitro* MRI (T2-weighted and T2-map) with a 3.0 Tesla clinical MR scanner. The sensitivity and specificity were evaluated based on the cellular uptake of four kinds of USPIO probes at the same dosage of iron concentration.

### Research results

The *in vitro* data showed that the double antibody-conjugated probe UAG had the best specificity in targeting Hepa1-6/GPC3 cells expressing AFP and GPC3 antigens (*vs* other USPIO probes including single antibody-labeled and unlabeled USPIOs). The iron Prussian blue staining and quantitative T2-map MRI analysis showed that, compared with UA, UG, and U, the uptake of the double-targeting UAG probe demonstrated a 23.3% (*vs* UA), 15.4% (*vs* UG), and 57.3% (*vs* U) increased Prussian stained cell percentage and a 14.93% (*vs* UA), 9.38% (*vs* UG), and 15.3% (*vs* U) reduction of T2 relaxation time, respectively. Such bi-specific probe might have the potential to overcome tumor heterogeneity with enhanced sensitivity and HCC specificity. Meanwhile, the coupling of two antibodies did not influence the magnetic performance of USPIO and the relatively small hydrodynamic size ( $59.60 \pm 1.87$  nm) of the double antibody-conjugated USPIO probe makes it a viable candidate for use in MHCC MRI *in vivo*, as they are slowly phagocytosed by macrophages. AFP and GPC3 were chosen based on clinical considerations. However, the cytoplasmic expression of AFP raised the complexity of the study in terms of probe internalization. The exact internalization route for such cytoplasmic antigen-targeted probes and whether the secreted AFP proteins contribute to probe internalization require detailed studies in the future.

### Research conclusions

The iron Prussian blue staining assay and *in vitro* MRI results confirmed that the bi-specific probe presents enhanced targeting efficiency and MRI sensitivity to HCC cells than singly- or non-targeted USPIO. Therefore, it implies that the multi-targeting strategy may be potentially applied in MRI probe design to enhance the malignant tumor recognition and MRI detection efficiency of MHCC for animal experiments and early clinical diagnosis.

### Research perspectives

The current research utilized monoclonal antibodies against AFP and GPC3 to ensure the specificity and purity. In future *in vivo* studies or investigation of cytoplasmic targeting by

USPIO, small antibody fragments possessing smaller molecular weights might be more effective. In addition, to further reduce non-specific uptake of USPIO by the reticuloendothelial system or mononuclear phagocytic system, surface modifications, such as hydrophilic PEG coatings for the USPIO, could be also considered in the *in vivo* experiments.

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## Basic Study

# Effect of NLRC5 on activation and reversion of hepatic stellate cells by regulating the nuclear factor- $\kappa$ B signaling pathway

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**Author contributions:** Zhang YZ, Yao JN, and Zhang LF designed the research; Zhang YZ, Yao JN, and Wang CF performed the research; Zhang LF and Wang CF contributed new reagents and analytic tools; Zhang XX and Gao B analyzed the data; Zhang YZ, Yao JN, Zhang LF, Wang CF, Zhang XX, and Gao B wrote the paper.

### Institutional review board

**statement:** The study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University.

### Institutional animal care and use

**committee statement:** The study was approved by the institutional animal care and use committee of the First Affiliated Hospital of Zhengzhou University.

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

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

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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## Abstract

### BACKGROUND

The formation of liver fibrosis is mainly caused by the activation of hepatic stellate cells (HSCs) and the imbalance of extracellular matrix (ECM) production and degradation. The treatment of liver fibrosis mainly includes removing the cause, inhibiting the activation of HSCs, and inhibiting inflammation. NOD-like receptor (NLR) family, caspase activation and recruitment domain (CARD) domain containing 5/NOD27/CLR16.1 (NLRC5) is a highly conserved member of the NLR family and is involved in inflammation and immune responses by regulating various signaling pathways such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling. It has been found that NLRC5 plays an important role in liver fibrosis, but its specific effect and possible mechanism remain to be fully elucidated.

### AIM

To investigate the role of NLRC5 in the activation and reversion of HSCs induced with transforming growth factor- $\beta$  (TGF- $\beta$ ) and MDI, and to explore its relationship with liver fibrosis.

### METHODS

A total of 24 male C57BL/6 mice were randomly divided into three groups, including normal, fibrosis, and recovery groups. Twenty-four hours after a liver fibrosis and spontaneous reversion model was established, the mice were sacrificed and pathological examination of liver tissue was performed to observe the degree of liver fibrosis in each group. LX-2 cells were cultured *in vitro* and treated with TGF- $\beta$ 1 and MDI. Real-time quantitative PCR (qPCR) and Western blot were used to analyze the expression levels of NLRC5,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and collagen type I alpha1 (Col1a1) in each group. The activity of NF- $\kappa$ B in each group of cells transfected with NLRC5-siRNA was detected.

### RESULTS

Compared with the normal mice, the expression level of NLRC5 increased

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significantly ( $P < 0.01$ ) in the fibrosis group, but decreased significantly in the recovery group ( $P < 0.01$ ). In *in vitro* experiments, the content of NLRC5 was enhanced after TGF- $\beta$ 1 stimulation and decreased to a lower level when treated with MDI ( $P < 0.01$ ). The expression of  $\alpha$ -SMA and Col1a1 proteins and mRNAs in TGF- $\beta$ 1-mediated cells was suppressed by transfection with NLRC5-siRNA ( $P < 0.01$ ). Western blot analysis showed that the expression of NF- $\kappa$ B p65 protein and phosphorylated I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ) was increased in the liver of mice in the fibrosis group but decreased in the recovery group ( $P < 0.01$ ), and the protein level of nuclear p65 and p-I $\kappa$ B $\alpha$  was significantly increased after treatment with NLRC5-siRNA ( $P < 0.01$ ).

## CONCLUSION

NLRC5 may play a key role in the development and reversal of hepatic fibrosis through the NF- $\kappa$ B signaling pathway, and it is expected to be one of the clinical therapeutic targets.

**Key words:** NLRC5; Hepatic stellate cells; Liver fibrosis; Recovery

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**Core tip:** Liver fibrosis is a pathological tissue repair process characterized by excessive deposition of extracellular matrix in the liver, accompanied by inflammation, and eventually progresses to cirrhosis. Several recent reports have shown that effective treatment can reverse liver fibrosis, which is associated with inactivation of hepatic stellate cells (HSCs) and multiple signaling pathways. NOD-like receptor (NLR) family, caspase activation and recruitment domain (CARD) domain containing 5/NOD27/CLR16.1 (NLRC5) is the largest member of the NLR family and is highly expressed in immune tissues or organs such as the spleen, lung, thymus, and liver, mediating inflammation inhibition and antiviral response. This study aimed to investigate the role of NLRC5 in activating and devitalization of HSCs and its mechanism. The results demonstrate that NLRC5 may be involved in the development and reversal of liver fibrosis by negative regulation of nuclear factor- $\kappa$ B.

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## INTRODUCTION

Liver fibrosis is a common consequence of long-term liver repair after injury caused by different etiologies<sup>[1-3]</sup>. The condition of liver fibrosis assumes the chronic process and continually progresses in the presence of injury factors. Ultimately, it will lead to a series of serious complications and increase the mortality rate related to liver cirrhosis<sup>[4,5]</sup>. With the gradual understanding of the fibrosis mechanism, a series of treatment measures such as removing the cause and inhibiting the activation of hepatic stellate cell (HSC) have been applied to clinical therapy<sup>[6-9]</sup>. Several recent reports have shown that frequent activation of HSCs for a long time will lead to extensive deposition of collagen fibers, resulting in liver fibrosis and liver dysfunction<sup>[10]</sup>. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) plays an important role in the activation of HSCs and is considered to be one of the most important fibrotic factors because it can inhibit extracellular matrix (ECM) degradation while promoting ECM synthesis<sup>[11-14]</sup>. It is well known that clearly knowing genes related to hepatic fibrosis, blocking the persistent damage of the liver, and then reversing liver fibrosis can be helpful in the treatment of this disease. The reversal of liver fibrosis is controversial for a long time. Studies in recent years, however, have found that liver fibrosis is reversible, and even patients with advanced liver fibrosis may return to normal, which has a correlation with the degradation of ECM and various signaling pathways<sup>[15-21]</sup>. Moreover, Abidali *et al*<sup>[22]</sup> confirmed that rat liver fibrosis can be automatically reversed after stopping the injection of carbon tetrachloride (CCl<sub>4</sub>), but



the possible mechanism of progression and reversal of liver fibrosis was not analyzed. Our previous study found that NLRC5 protein levels fluctuated abnormally during progression and reversal of liver fibrosis. As the largest member of the NOD-like receptor (NLR) family, NLRC5 is widely expressed in various tissues and cell lines of humans and mice, but is mainly concentrated in immune cells and immune-related tissues<sup>[23,24]</sup>. Current studies on NLRC5 are mostly focused on the regulation of major histocompatibility complex (MHC) I gene expression and the participation in the antiviral innate immune response<sup>[25,26]</sup>. In the study by Catalano *et al.*<sup>[27]</sup>, NLRC5 can negatively regulate the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway by inhibiting the phosphorylation of  $\kappa$ B-inhibiting protein kinase alpha antibody (IKK $\alpha$ ) and  $\kappa$ B-inhibiting protein kinase beta antibody (IKK $\beta$ ), while the activation of this signaling pathway is closely related to the activation of HSCs. These results suggest that NLRC5 is involved in the development of liver fibrosis, however, there are few reports on the role of NLRC5 in liver fibrosis. Therefore, this study aimed to investigate the role and mechanism of NLRC5 in HSC activation and reversal, and to explore its relationship with liver fibrosis to evaluate its clinical application value.

## MATERIALS AND METHODS

### Animal model

A total of 24 male C57BL/6 mice weighing 16-18 g were provided as the CCl<sub>4</sub> liver injury model by the First Affiliated Hospital of Zhengzhou University. Animals were randomly divided into the following groups: normal mice (normal group), mice with liver fibrosis (fibrosis group), and mice with reversal of liver fibrosis (recovery group). Before the experiments, they were placed in a clean animal room at 24 °C with free access to water and food for one week. Fibrosis was induced in mice by subcutaneous administration of CCl<sub>4</sub> (10% CCl<sub>4</sub> in olive oil, Shanghai Zhanyun Chemical Co., Ltd.) at a dose of 2 mL/kg, 3 times a week for 6 wk, and in the recovery group, it was discontinued for another 4 wk. The normal group was injected with the same dose of olive oil for the same treatment time. The mice were sacrificed at 24 h after the last injection of CCl<sub>4</sub> and liver tissues were excised to confirm the successful establishment of the fibrosis model.

### Cell culture

Human hepatic stellate cell line LX-2 (Shanghai Yaji Biotechnology Co., Ltd.) was cultured in DMEM medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 IU/mL penicillin/streptomycin, and 1% glutamine. Then, the medium was placed in an incubator with 5% CO<sub>2</sub> and 70%-80% humidity at 37 °C. In accordance with the routine culture, the medium was changed daily. Digestion and passage were performed until 80% confluence of cells. Cells in the logarithmic growth phase were used in experiments. Four groups of LX-2 cells were studied: (1) TGF- $\beta$ 1: treated with recombinant human 5 ng/mL TGF- $\beta$ 1 (PeproTech, United States) and cultivated for 24 h; (2) Control: normal cells; and (3) TGF- $\beta$ 1 + MDI: MDI (50  $\mu$ L 3-isobutyl-1-methylxanthine + 10  $\mu$ L dexamethasone + 576.7  $\mu$ L insulin, Sigma, United States) was added into culture of LX-2 cells for 48 h following treatment with 2.5 ng/mL TGF- $\beta$ 1.

### Histological examination

The fresh liver tissues of mice were removed and fixed in fixative solution prepared (10% formalin, Bouin's fixative) to denature and coagulate tissue and cellular proteins. Then, the tissue blocks were dehydrated using graded ethanol, cleared in xylene, embedded in paraffin, and sectioned at 5  $\mu$ m thickness. After xylene dewaxing and gradient ethanol hydration, hematoxylin and eosin staining and Masson staining (Shanghai Ruchuang Biotechnology Co., Ltd.) were performed to observe the histopathological changes of the liver.

### Immunohistochemistry

Routine xylene dewaxing, gradient ethanol hydration, and citric thermal remediation for 15 min were done and paraffin-embedded liver tissue was handled with 3% H<sub>2</sub>O<sub>2</sub> for 10 min to block endogenous peroxidase activity. Then, normal sheep serum was used to seal up the nonspecific sites. After that, the sections were incubated with primary antibody against  $\alpha$ -SMA (dilution, 1:400; Shanghai Ru Chuang Biotechnology Co., Ltd.) overnight at 4 °C, followed by incubation with a biotinylated secondary antibody (Shanghai Ruchuang Biotechnology Co., Ltd.) for 60 min at room temperature. The sections were stained with DAB, counterstained with haematoxylin, finally mounted, and observed under a microscope.

### SiRNA transfection

The design and synthesis of specific siRNA for NLRC5 were assisted by Shanghai Hengfei Biotechnology Co., Ltd. The NLRC5-siRNA sense sequence was 5'-GGG ACTGAGAGCTTTGTAT-3', and the antisense sequence was 5'-CGC ACCCTAGACTGAAA-3'. The sense sequence of the NLRC5-siRNA negative control was 5'-UUCUCCGAACGUGUCACGUTT-3', and the antisense was 5'-ACG UGACACGUUCGGAGAATT-3'. LX-2 cells were cultured in DMEM medium with 10% FBS at a density of  $2 \times 10^5$  cells/mL. The diluted Lipofectamine™ 2000 (Invitrogen, United States) were mixed with the diluted siRNA oligomer and incubated for 20 min, then the mixture was added into the medium. The medium was changed at 6 h post-transfection, and TGF- $\beta$ 1 was added at a concentration of 10 ng/mL to culture for an additional 48 h. Q-PCR and Western blot were used to detect the expression of NLRC5, and normal LX-2 cells transfected with NLRC5-siRNA negative control were used as controls.

### Real-time fluorescent quantitative PCR

Total RNA was extracted from liver tissue and LX-2 cells using Trizol reagent (Invitrogen, United States) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Dalian Bao Bioengineering Co., Ltd.), and the target gene was detected using an SYBR Green Fluorescence Quantitation Kit (Dalian Bao Bioengineering Co., Ltd.). The 25- $\mu$ L PCR reaction system consisted of 1  $\mu$ L of upstream and downstream primers (1  $\mu$ mol/L), 1  $\mu$ L of cDNA product, 2.5  $\mu$ L of Taq 10  $\times$  Buffer, 1  $\mu$ L of Taq DNA polymerase, and 3  $\mu$ L of dNTP mixture, and the remaining volume was supplemented with deionized water. The amplification parameters were pre-denaturation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 5 s, annealing at 59 °C for 30 s, and extension at 72 °C for 1 min.  $\beta$ -actin was used as an internal reference gene, and the Ct value of each sample was analyzed. The primer sequences were designed according to the Genbank database and using Prime 5 software, and the sequences used are as follows: NLRC5 forward, 5'-CTATCAACTGCCCTTCCACAAT-3' and reverse, 5'-TCTCTATCTGCCACAGCC-TAC-3';  $\alpha$ -SMA forward, 5'-CTATTCCTTCGTGACTACT-3' and reverse, 5'-ATGCTGTTATAGGTGGTGGT-3'; Col1a1 forward, 5'-CCCGGGTTTCAGAG-ACAACCTTC-3' and reverse, 5'-TCCACATGCTTTATCCAGCAATC-3';  $\beta$ -actin forward, 5'-GAGGCACTCTTCCAGCCTTC-3' and reverse, 5'-GGATGTC CACGTCACACTTC-3'.

### Western blot

Nuclear and cytoplasmic proteins were obtained from mouse liver tissues and cells using radioimmunoprecipitation assay (RIPA) lysis buffer and PMSF (100:1 Beijing Saibaisheng Gene Technology Co., Ltd.). The protein contents of the samples were determined by the bicinchoninic acid (BCA, Beijing Saibaisheng Gene Technology Co., Ltd.) method. Twenty micrograms of protein samples were separated by 10% SDS-PAGE, and transferred to polyvinylidene fluoride membranes. Following blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated with primary antibodies against NLRC5 (Shanghai Youningwei Biotechnology Co., Ltd.) at 1:3000 dilution,  $\alpha$ -SMA (Shanghai Gefan Biotechnology Co., Ltd.) at 1:3000 dilution, Col1a1 (Shanghai Gefan Biotechnology Co., Ltd.) at 1:3000 dilution,  $\beta$ -actin (Santa Cruz) at 1:1000 dilution, I $\kappa$ B $\alpha$  (Cell Signal) at 1:2000 dilution, p-I $\kappa$ B $\alpha$  (Cell Signal) at 1:2000 dilution, and p65 (Cell Signal) at 1:2000 dilution overnight at 41 °C. After washing with TBST, diluted IgG antibody conjugated with horseradish peroxidase (1:2000 Santa Cruz) was added to incubate for 2 h at room temperature. The membranes were developed with an enhanced chemiluminescence detection kit (Beijing Saibaisheng Gene Technology Co., Ltd.).

### Statistical analysis

Statistical analyses were performed using SPSS 19.0 software, and the experimental data are expressed as the mean  $\pm$  standard deviation. One-way ANOVA was applied to determine the significant difference, and the differences between groups were tested using the LSD test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Histopathological results of liver fibrosis

Fibrosis was determined by HE staining and Masson staining. After 6 wk of CCl<sub>4</sub> induction, the mouse liver become coarse and dark gray in color. The liver tissue showed relative swelling, punctate necrosis, and regeneration. Infiltration of inflammatory cells, fibrous hyperplasia, steatosis, and ballooning changes were found in the central vein and the portal area, and the structure of partial hepatic lobules was

not clear. In contrast, the liver of the normal mice was red in color and well marginated, the structure of the hepatic lobule was normal and integral, the cords of liver cells were arranged orderly, and there was no degeneration, necrosis, or inflammatory cells infiltration. In the recovery group, the contour of liver tissue was fine and the structure became normal (Figure 1). In addition, the results of immunohistochemistry showed that the expression of  $\alpha$ -SMA was high in the experimental group, distributed in the cytoplasm of hepatocytes, and aggregated in the portal area, but only a little was observed in the normal and recovery groups (Figure 2). The relative protein and mRNA levels of  $\alpha$ -SMA and Col1a1 were increased in LX-2 cells under TGF- $\beta$ 1 stimulation ( $P < 0.01$ ), while decreased after MDI treatment ( $P < 0.05$ ). As shown in Figure 3, the successful establishment and reversal of fibrosis model were confirmed.

### **Expression of NLRC5 in liver tissue and LX-2 cells**

To investigate the differential expression of NLRC5 during fibrosis and reversal model, Western blot and qPCR were used to analyze the relative protein and mRNA expression levels of NLRC5. In contrast with the normal group, the expression level of NLRC5 increased significantly in the fibrosis group ( $P < 0.01$ ), but significantly decreased in the recovery group ( $P < 0.01$ ) (Figure 4). For the *in vitro* experiments, the expression level of NLRC5 was enhanced after TGF- $\beta$ 1 stimulation ( $P < 0.01$ ), and decreased to a lower level when treated with MDI ( $P < 0.01$ ) (Figure 5).

### **Effect of NLRC5-siRNA transfection on the expression of NLRC5 in LX-2 cells**

Western blot was applied to confirm whether NLRC5-siRNA was successfully transfected into LX-2 cells. The results showed that the NLRC5 protein level in LX-2 cells transfected with NLRC5-specific siRNA was obviously lower compared with control cells and NLRC5-siRNA negative control ( $P < 0.01$ ) (Figure 6).

### **Effect of NLRC5 on the expression of $\alpha$ -SMA and Col1a1 in LX-2 cells**

$\alpha$ -SMA is one of the markers of activated HSCs. Q-PCR and Western blot were used to analyze the relationship between the change of NLRC5 in LX-2 cells and the expression of  $\alpha$ -SMA. As shown in Figure 7, it was observed that the down-regulation of NLRC5 decreased the expression levels of  $\alpha$ -SMA protein and mRNA in LX-2 cells stimulated with TGF- $\beta$ 1 ( $P < 0.01$ ), and Col1a1 expression was also suppressed by transfection of NLRC5-siRNA ( $P < 0.01$ ).

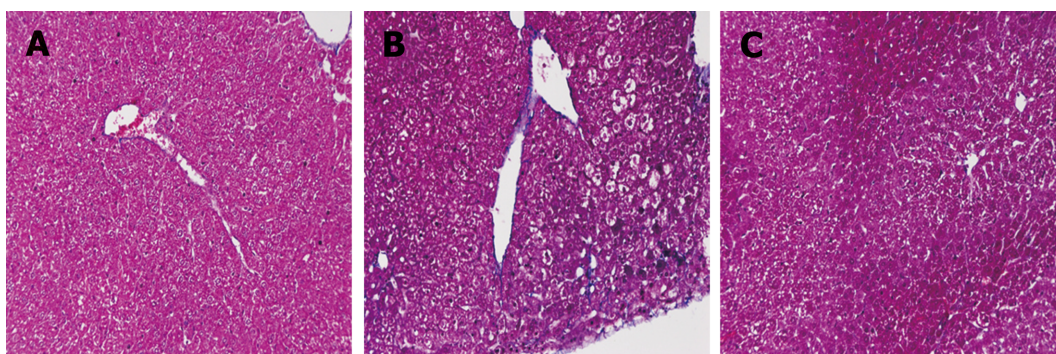
### **Effect of NLRC5 on NF- $\kappa$ B activity in hepatic fibrosis**

Western blot analysis showed that the expression of p65 and p-I $\kappa$ B $\alpha$  increased in the liver of mice in the fibrosis group ( $P < 0.01$ ), but decreased in the recovery group ( $P < 0.05$ ). There was no significant change in total protein of I $\kappa$ B $\alpha$  (Figure 8). To detect the relationship between NLRC5 and the NF- $\kappa$ B signaling pathway during the activation of HSCs, the effect of NLRC5-siRNA on LX-2 cells induced with TGF- $\beta$ 1 was investigated. The results showed that the protein levels of nuclear p65 and p-I $\kappa$ B $\alpha$  were significantly increased after treatment with NLRC5-siRNA ( $P < 0.01$ ), while the level of cytoplasmic p65 was decreased ( $P < 0.05$ ), and the expression of I $\kappa$ B $\alpha$  protein was almost unchanged (Figure 9).

## **DISCUSSION**

Hepatic fibrosis is a common pathological change in chronic liver injury, involving multiple types of molecules, cells, and tissues<sup>[28]</sup>. If treated not appropriately, the condition would develop to histological cirrhosis or hepatocellular carcinoma, and result in serious consequences. From the pathological mechanism, liver fibrosis is mainly induced by the accumulation of ECM protein in the hepatic lobules and portal area. Excessive deposition of ECM destroys the normal structure of the liver, leading to hepatic function damage<sup>[29,30]</sup>. In this process, activation of HSCs is the key point in hepatic fibrosis. Phenotypic modulation of activated HSCs is enhanced, then these cells are transformed into myofibroblast-like cells, express  $\alpha$ -SMA, produce ECM, and accelerate liver fibrosis following hepatic damage<sup>[31-33]</sup>. Meanwhile, TGF- $\beta$ 1 inhibits HSC apoptosis by means of autocrine and paracrine mechanisms, induces matrix protein expression, and therefore becomes one of the most important cytokines in the process of liver fibrosis<sup>[34]</sup>. Since the promoter of the TGF- $\beta$ 1 activating factor tissue transglutaminase gene contains a binding site for NF- $\kappa$ B, the synthesis of TGF- $\beta$ 1 is regulated by NF- $\kappa$ B<sup>[35,36]</sup>. Additionally, experimental studies have shown that NF- $\kappa$ B can amplify the liver inflammatory reaction by enhancing the expression of some factors related to HSC activation including inflammatory factors (IL-1, TNF- $\alpha$ , and IL-6), cell adhesion factors, and transforming growth factors, aggravating the disease<sup>[37-40]</sup>.



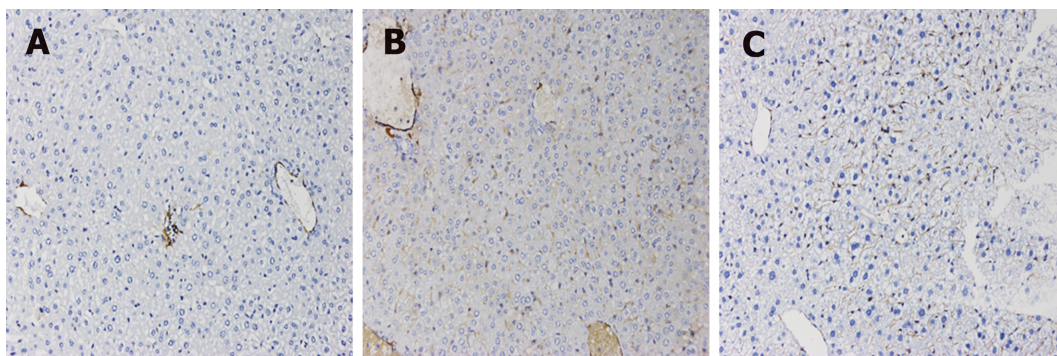


**Figure 1** Masson staining for assessment of liver tissue in each group (×200). A: Liver tissue from normal mice (normal group); B: Mice with liver fibrosis (fibrosis group); C: Reversal of liver fibrosis in mice (recovery group).

Currently, NLRC5 is a new direction in the study of the NF- $\kappa$ B signaling pathway. It has been confirmed that NLRC5 plays an important role in regulating the NF- $\kappa$ B signaling pathway, type I interferon (INF-I) signaling pathway, and MHC-I gene expression<sup>[41,42]</sup>. As a member of the NLR family, NLRC5 can respond to various pathogens and intracellular danger signals and participates in the body's innate and adaptive immune responses. It is worth mentioning that NLRC5 is related to immune responses, besides that it may be a key regulator during development and reversal of hepatic fibrosis<sup>[43]</sup>, but little is known about the role of NLRC5 in hepatic fibrosis. Therefore, this article analyzed the relationship between NLRC5 and hepatic fibrosis and its related mechanism, in order to evaluate its clinical application value.

In this study, the expression levels of NLRC5 in liver tissue and LX-2 cells were analyzed to explore the role of NLRC5 in liver fibrosis, and the results showed that the expression of NLRC5 in the fibrosis group was significantly higher than that in the normal and recovery groups. Moreover, NLRC5 was increased in LX-2 cells treated with TGF- $\beta$ 1 while decreased in cells following treatment with MDI as compared with the control cells. Based on these results, it was suggested that NLRC5 may play an important role during the occurrence and reversal of liver fibrosis. Transfection with NLRC5-siRNA in activated LX-2 cells can reduce the expression of  $\alpha$ -SMA and Colla1, suggesting a decrease in the number of activated HSCs.

Large animal experiments indicated that the major pathophysiological mechanism of HSC activation is transcriptional activation mediated by NF- $\kappa$ B<sup>[44,45]</sup>. The expression and secretion of various inflammatory factors and adhesion molecules can be induced and involved in the formation of liver fibrosis during increased NF- $\kappa$ B activity. In the resting state, p65 subunit binds to the I $\kappa$ B monomer and is located in the cytoplasm, and NF- $\kappa$ B in the nucleus is deficient. When suffering the outsider incitement, p65 translocates into the nucleus and activates NF- $\kappa$ B through the classical pathway following the phosphorylation and degradation of I $\kappa$ B $\alpha$ <sup>[46]</sup>. The results of this study showed that the expression levels of p65 and p-I $\kappa$ B $\alpha$  in the nucleus were significantly increased after NLRC5-siRNA treatment, suggesting that NLRC5 may be involved in liver fibrosis by negative regulation of NF- $\kappa$ B. In the study by Chang G *et al*<sup>[47]</sup>, the high expression of NLRC5 protein in mouse hepatic fibrosis after activation of the NF- $\kappa$ B signaling pathway was presented. NLRC5 protein, because of its high expression level and large relative molecular mass, can inhibit the binding of NEMO to IKK $\alpha$  and IKK $\beta$  and down-regulate the expression of nuclear p65 and p-I $\kappa$ B $\alpha$ , and it is also activated by IKK $\alpha$  and IKK $\beta$ , forming a negative regulation cycle<sup>[48,49]</sup>. The inactive NF- $\kappa$ B stays within the cytoplasm, close to I $\kappa$ B family members in the inhibitory protein family. The interaction of NLRC5 with IKK $\alpha$  and IKK $\beta$  blocks the phosphorylation of the inhibitory protein I $\kappa$ B and eventually inhibits the activation of NF- $\kappa$ B signaling pathway<sup>[50]</sup>. More importantly, it was found that specific knockout of NLRC5 not only enhanced NF- $\kappa$ B and type I interferon signaling and target gene expression, but also modulated antiviral immune responses of multicellular lines and primary cells in a study of NLRC5 and NF- $\kappa$ B<sup>[51]</sup>. With conserved biological functions in humans and mice, as well as in various cell types, NLRC5 may be critical in the maintenance of immune homeostasis, particularly in regulation of innate immune responses<sup>[52]</sup>. However, the relationship between NLRC5 and the NF- $\kappa$ B signaling pathway and its roles in many types of diseases have yet to be determined. According to the results of this study, we infer that NLRC5 itself is induced by NF- $\kappa$ B-dependent mechanisms in LX-2 cells. Activation of TGF- $\beta$ 1 increases the expression of phosphorylated I $\kappa$ B $\alpha$ , stimulates the translocation of p65 from the cytoplasm to nucleus, and promotes the expression of NLRC5 in HSCs. The interaction of NLRC5

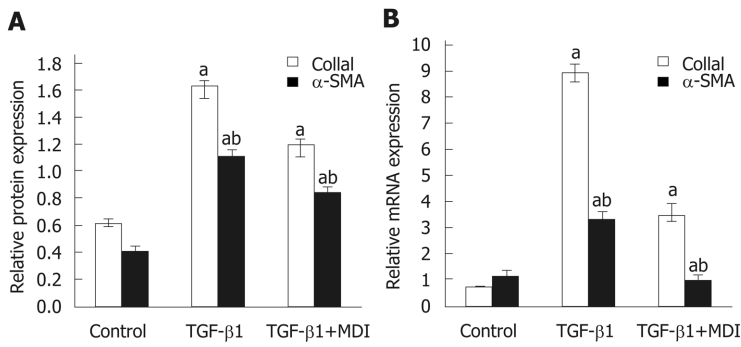


**Figure 2** Immunohistochemical staining for  $\alpha$ -SMA in liver tissue in each group ( $\times 200$ ). A: Liver tissue from normal mice (normal group); B: Mice with liver fibrosis (fibrosis group); C: Reversal of liver fibrosis in mice (recovery group).

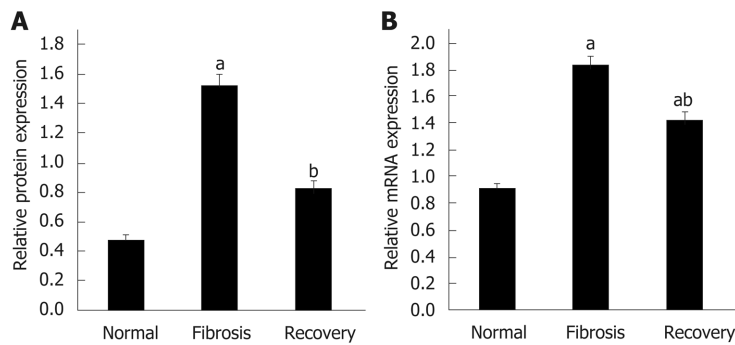
and NF- $\kappa$ B accelerates the development of liver fibrosis. There are still some limitations that need to be improved in this paper. The research on the NF- $\kappa$ B signaling pathway is not deep enough and whether there are other signaling pathways involved requires further research.

In conclusion, NLRC5 may participate in the process of HSC activation and ECM synthesis through the NF- $\kappa$ B signaling pathway. It can intervene in the key processes related to liver fibrosis, which provides a theoretical basis for clinical treatment of liver fibrosis.

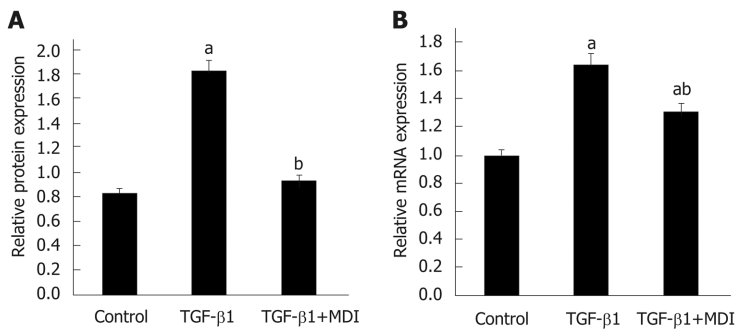




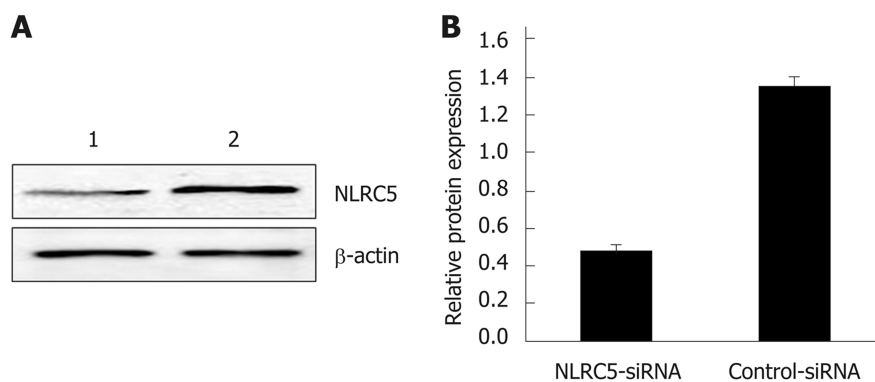
**Figure 3** Expression of  $\alpha$ -SMA and Col1 $\alpha$ 1 in LX-2 cells. A: Relative mRNA expression; B: Quantification of Western blot. <sup>a</sup> $P < 0.05$  vs control; <sup>b</sup> $P < 0.05$  vs transforming growth factor- $\beta$ 1. TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1.



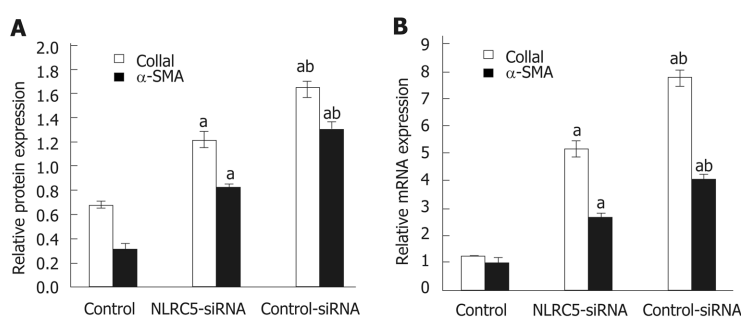
**Figure 4** Expression level of NLRC5 in each group of mice. A: Relative mRNA expression; B: Quantification of Western blot. <sup>a</sup> $P < 0.05$  vs normal; <sup>b</sup> $P < 0.05$  vs fibrosis.



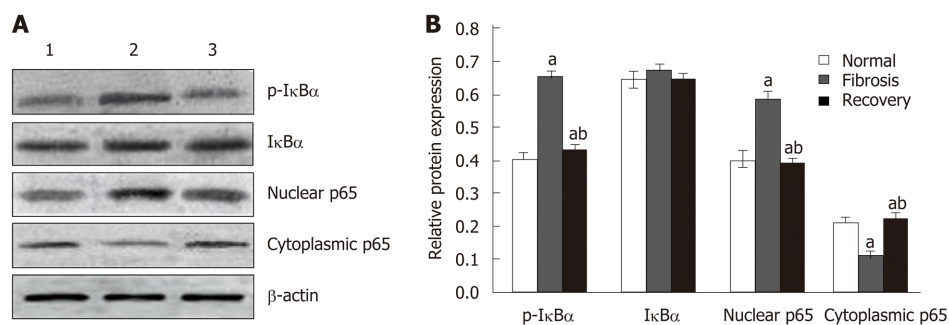
**Figure 5** Expression level of NLRC5 in LX-2 cells. A: Relative mRNA expression; B: Quantification of Western blot. <sup>a</sup> $P < 0.05$  vs control; <sup>b</sup> $P < 0.05$  vs transforming growth factor- $\beta$ 1. TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1.



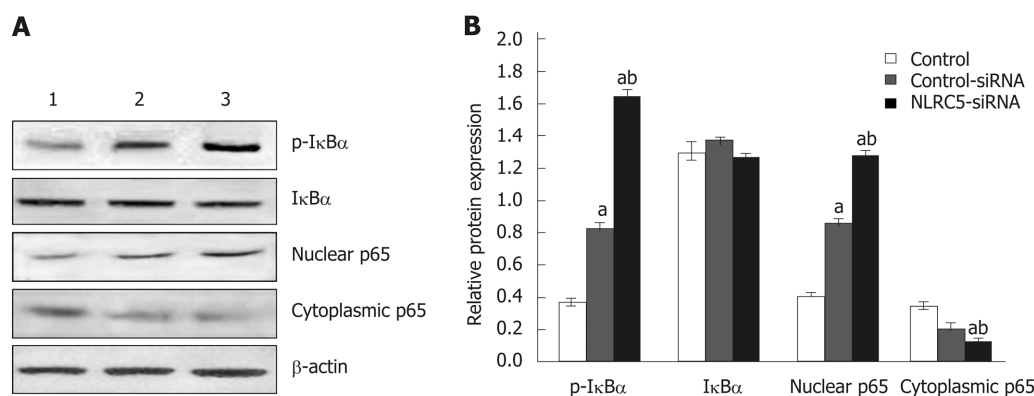
**Figure 6** Effect of NLRC5 knockdown in LX-2 cells. A: Western blot; B: Quantification of Western blot. Lane 1: LX-2 cells transfected with NLRC5-siRNA; Lane 2: LX-2 cells transfected with control-siRNA;  $^aP < 0.05$  vs control-siRNA.



**Figure 7** Effect of NLRC5 knockdown on α-SMA and Col1a1 expression in LX-2 cells. A: Relative mRNA expression; B: Quantification of Western blot.  $^aP < 0.05$  vs control;  $^bP < 0.05$  vs NLRC5-siRNA.



**Figure 8** Effect of NLRC5 on nuclear factor-κB activity in mice hepatocytes. A: Western blot; B: Quantification of Western blot. Lane 1: Normal mice (normal group); Lane 2: Mice with liver fibrosis (fibrosis group); Lane 3: Reversal of liver fibrosis in mice (recovery group);  $^aP < 0.05$  vs normal;  $^bP < 0.05$  vs fibrosis.



**Figure 9** Effect of NLRC5 on nuclear factor-κB activity in LX-2 cells. A: Western blot; B: Quantification of Western blot. Lane 1: Control; Lane 2: LX-2 cells transfected with NLRC5-siRNA; Lane 3: LX-2 cells transfected with control-siRNA; <sup>a</sup> $P < 0.05$  vs control; <sup>b</sup> $P < 0.05$  vs NLRC5-siRNA.

## ARTICLE HIGHLIGHTS

### Research background

Continuous progression of liver fibrosis is the key to the development of chronic liver disease to cirrhosis. The nuclear factor-κB (NF-κB) signaling pathway is closely related to the formation and reversion of hepatic fibrosis. It has been found that NLRC5 is involved in the development of liver fibrosis by regulating the NF-κB signaling pathway and some studies suggest that NLRC5 is a key regulator of liver fibrosis and its reversal, but the role of NLRC5 in liver fibrosis remains unclear.

### Research motivation

NLRC5 is highly expressed in immune tissues or organs and involved in the regulation of innate and adaptive immunity by inducing inflammation and cell death. Researches have shown that it may play an important role in the activation and inactivation of hepatocytes, but the research on the mechanism of action fell far behind the immunological study. Our study aimed to investigate the role and mechanism of NLRC5 in liver fibrosis to evaluate its clinical application value.

### Research objectives

In this study, we analyzed the expression levels of NLRC5 in liver tissue and LX-2 cells and the activity of NF-κB in hepatic fibrosis after treatment with NLRC5-siRNA. The purpose of this study was to explore the relationship between NLRC5 and the NF-κB signaling pathways during the development and reversal of hepatic fibrosis.

### Research methods

Eight-week-old male C57BL/6 mice were randomly divided into groups to establish liver fibrosis and its reversal model. Meanwhile, human hepatic stellate cell (HSC) line LX-2 was cultured *in vitro* and treated with transforming growth factor-β1 (TGF-β1) and MDI to activate and inactivate the cells. The degree of liver fibrosis and the expression of NLRC5 in mouse tissues and LX-2 cells were detected by qPCR and Western blot. After interfering with NLRC5 by siRNA, the activity of NF-κB in liver fibrosis was detected.

### Research results

The expression level of NLRC5 was higher in liver tissue of fibrosis mice and activated HSCs, but decreased in mice with hepatic fibrosis with spontaneous reversion and inactivated HSC cells ( $P < 0.01$ ). After treatment with NLRC5-siRNA, the activity of the NF-κB signaling pathway was increased in the liver of fibrosis mice and activated HSCs ( $P < 0.05$ ).

### Research conclusions

NLRC5 may play a key role in regulating the progression and reversal of liver fibrosis by negatively regulating the NF-κB signaling pathway, and it is expected to be one of the clinical therapeutic targets.

### Research perspectives

NLRC5 plays a physiologically important role in maintaining immune homeostasis, particularly in regulating innate immune responses. Exploring the role and mechanism of NLRC5 and NF-κB in liver fibrosis can provide an important reference for the treatment of liver fibrosis.

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## Basic Study

# Freeze-dried Si-Ni-San powder can ameliorate high fat diet-induced non-alcoholic fatty liver disease

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## Abstract

### BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease worldwide. However, to date, there is no ideal therapy for this disease.

### AIM

To study the effects of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD in mice.

### METHODS

Twenty-four male C57BL/6 mice were randomized into three groups of eight. The control group (CON) was allowed *ad libitum* access to a normal chow diet. The high fat diet group (FAT) and Si-Ni-San group (SNS) were allowed *ad libitum* access to a high fat diet. The SNS group was intragastrically administered Si-Ni-San freeze-dried powder (5.0 g/kg) once daily, and the CON and FAT groups were intragastrically administered distilled water. After 12 wk, body weight, liver index, visceral fat index, serum alanine aminotransferase (ALT), portal lipopolysaccharide (LPS), liver tumor necrosis factor (TNF)- $\alpha$  and liver triglycerides were measured. Intestinal microbiota were analyzed using a 16S rDNA sequencing technique.

### RESULTS

Compared with the FAT group, the SNS group exhibited decreased body weight, liver index, visceral fat index, serum ALT, portal LPS, liver TNF- $\alpha$  and liver triglycerides ( $P < 0.05$ ). Intestinal microbiota analysis showed that the SNS group had different bacterial composition and function compared with the FAT group. In particular, *Oscillospira* genus was a bacterial biomarker of SNS group samples.

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## CONCLUSION

The beneficial effects of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD in mice may be associated with its anti-inflammatory and changing intestinal microbiota effects.

**Key words:** Nonalcoholic fatty liver disease; Si-Ni-San; High fat diet; Intestinal microbiota; Inflammation

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**Core tip:** We studied the effects of Si-Ni-San freeze-dried powder on high fat diet-induced nonalcoholic fatty liver disease (NAFLD) in mice. We found that Si-Ni-San freeze-dried powder ameliorated high fat diet-induced NAFLD in mice, and the mechanism of action of Si-Ni-San freeze-dried powder against NAFLD may be associated with its anti-inflammatory and changing intestinal microbiota effects. Our findings provide some useful information for therapy of NAFLD.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a condition of excess fat accumulation in the liver without significant alcohol consumption, and it consists of liver damage, ranging from steatosis to steatohepatitis, advanced fibrosis and cirrhosis. Obesity, hypertriglyceridemia, hyperglycemia, and type 2 diabetes are the best-known risk factors for NAFLD<sup>[1,2]</sup>. Besides these risk factors, plus-size clothing and sleep shortage may also be associated with NAFLD<sup>[3]</sup>.

Over recent decades, we have witnessed a markedly increasing incidence of NAFLD, and it has become one of the most common chronic liver diseases worldwide<sup>[4,5]</sup>. In the United States, the proportion of patients with this chronic liver diseases rose from 47%-75%<sup>[6]</sup>. With the prevalent use of hepatitis vaccine and unchanged lifestyle, we can foresee that this proportion will continue to rise in the future. Nonalcoholic steatohepatitis (NASH), a more severe form of NAFLD, can lead to cirrhosis, liver failure, hepatocellular carcinoma and liver-related death<sup>[7-9]</sup>. In addition, NAFLD is a risk factor for cardiovascular diseases, diabetes and chronic kidney disease, and it also causes a high level of non-liver-related mortality<sup>[10,11]</sup>. Although NAFLD poses a threat to human health, the exact pathogenesis of this disease remains unclear. Moreover, until now, there are no approved medications for NAFLD treatment. Despite guidelines<sup>[12,13]</sup> recommending lifestyle modification as the first line treatment for NAFLD, compliance is a challenge. Therefore, it is important to look for new drug therapies.

Si-Ni-San, first recorded by Zhong-Jing Zhang during the Eastern Han Dynasty, is a famous prescription of traditional Chinese medicine to coordinate the functions of liver and spleen, and it has been used in China for thousands of years. This prescription consists of four herbal medicines: Bupleuri Radix, Paeoniae Alba Radix, Aurantii Immaturus Fructus, and Honey-fried Licorice Root in equal proportions. Si-Ni-San can alleviate liver injury through protecting hepatocyte membranes, increasing NO release and facilitating apoptosis of liver-infiltrating cells<sup>[14]</sup>, and modified Si-Ni-San also has hepatoprotective effects<sup>[15]</sup>. Moreover, glycyrrhizin<sup>[16]</sup> and paeoniflorin<sup>[17]</sup>, two components of Si-Ni-San, have shown beneficial effects on NAFLD. Therefore, we hypothesize that Si-Ni-San may have some beneficial effects against NAFLD.

The human intestine harbors 10-100 trillion microorganisms, mainly bacteria, collectively referred to as the intestinal microbiota<sup>[18,19]</sup>. The intestinal microbiota carries 150-fold more genes than the human genome, and these vast number of genes endow our body with special functions that we have not acquired during evolution, such as digesting plant polysaccharides<sup>[20,21]</sup>. Although the homeostasis of the

intestinal microbiota is important for human health, dysbiosis of intestinal microbiota may cause disease. Previous studies have demonstrated that intestinal microbiota play a crucial role in the development of NAFLD<sup>[22-24]</sup>, and patients with NAFLD have a different intestinal microbiota composition compared with healthy controls<sup>[25]</sup>. After oral administration, herbal medicines are exposed to the intestinal microbiota, and interactions are inevitable. Emerging studies have found that some herbal medicines can change the composition of the intestinal microbiota, which is viewed as an underlying therapeutic mechanism of herbal medicines<sup>[26,27]</sup>. In contrast, for other herbal medicines, such as berberine<sup>[28]</sup> and hesperidin<sup>[29]</sup>, the intestinal microbiota play a critical role in mediating their therapeutic effects.

The purpose of the present study was to preliminarily investigate the therapeutic effect of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD in a mouse model, and its effect on the composition and function of the intestinal microbiota, which will provide us with a deeper understanding of the therapeutic mechanism of Si-Ni-San freeze-dried powder.

## MATERIALS AND METHODS

### *Preparation of Si-Ni-San freeze-dried powder*

Bupleuri Radix, Paeoniae Alba Radix, Aurantii Immaturus Fructus, and Honey-fried Licorice Root were purchased from Beijing Tongrentang (Beijing, China) and were authenticated by our team. The herbs were mixed at a mass ratio of 1:1:1:1, and the mixture (2000 g) was decocted with distilled water and then filtered. The filtrate was prepared by freeze-drying, and five major constituents in the freeze-dried powder were quantified by HPLC (Table 1).

### *Animals, diets, and treatments*

Twenty-four male 5-wk-old C57BL/6 mice (Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were acclimated for 1 wk at a temperature of 20–22 °C and humidity of 40%–45% in controlled rooms with an alternating 12-h light and dark cycle. After acclimation, mice were randomized into three groups of eight. The control group (CON) was allowed *ad libitum* access to a normal chow diet for 12 wk. The high fat diet group (FAT) and Si-Ni-San group (SNS) were allowed *ad libitum* access to a high fat diet for 12 wk. The composition of normal chow diet and high fat diet are shown in Table 2. SNS group mice were intragastrically administered Si-Ni-San freeze-dried powder (5.0 g/kg) once daily. The CON and FAT groups were intragastrically administered distilled water once daily. The study was approved by the Animal Ethics Committee of Hebei North University (No. 2016-1-0-06).

### *Liver index and visceral fat index assays*

After 12 wk treatment, all mice were anesthetized with ketamine (80 mg/kg) and xylazine (6 mg/kg). The liver, mesenteric fat, retroperitoneal fat and epididymal fat were isolated and weighed. Liver index was calculated as the ratio of liver to body weight. The visceral fat index was calculated as the ratio of visceral fat (mesenteric, retroperitoneal and epididymal fat) to body weight.

### *Hepatic lipid measurement*

Liver lipid was extracted as previously described<sup>[30]</sup>, and liver triglycerides were measured using a Triglyceride Reagent Kit (Dongou, Wenzhou, China). Frozen sections stained with Oil Red O were also used for hepatic lipid detection.

### *Biochemical assays*

When the mice were killed, blood samples were collected *via* cardiac puncture and centrifuged. Serum alanine aminotransferase (ALT) was measured by standard procedures. One milliliter of portal blood was collected for analysis by lipopolysaccharide (LPS) assay. The level of portal LPS was measured using a chromogenic limulus amoebocyte lysate test kit (Bokang, Zhanjiang, China) according to the manufacturer's instructions.

### *Liver TNF-α*

Portions of liver tissues were homogenized (100 mg/mL) in RIPA lysis buffer (Beyotime, Shanghai, China). Liver TNF-α was measured using Mouse TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, United States).

### *Intestinal microbiota analysis*

One week before the mice were killed, fecal samples were collected and stored at -80 °C. The total fecal DNA was extracted using QIAamp Fast DNA Stool Mini Kit

**Table 1** Five major constituents in Si-Ni-San freeze-dried powder

Compound	Saikoside A	Paeoniloricin	Naringin	Hesperidin	Licorice acid
Contents <sup>1</sup> In mg/g	89.5 ± 0.02	458.2 ± 0.03	143.4 ± 0.03	138.3 ± 0.02	140.3 ± 0.01

<sup>1</sup>Data expressed as mean ± SD (*n* = 5).

(QIAGEN, Valencia, CA, United States). V4 hypervariable region of 16S rRNA genes was amplified using specific primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3'). PCR products were mixed in equidensity ratios. Sequencing libraries were prepared using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, United States). The library was sequenced on an Illumina HiSeq2500 platform and paired-end reads were generated.

QIIME pipeline (1.9.1) was used to process and analyze the raw data<sup>[31]</sup>. The main scripts used in our study were as follows: `join_paired_ends.py`, `split_libraries_fastq.py`, `pick_open_reference_otus.py` and `core_diversity_analyses.py`. Operational taxonomic units (OTUs) were clustered at 97% similarity, and sequences were taxonomically assigned against the Greengenes database (gg\_13\_8). The results of  $\alpha$  diversity,  $\beta$  diversity and bacterial taxonomy were generated by `core_diversity_analyses.py` script. We used the linear discriminant analysis (LDA) effect size (LEfSe) method to identify bacterial biomarkers in different groups<sup>[32]</sup>. We predicted the bacterial functions of different groups. For functional prediction, we reclustered sequences into OTUs (97% similarity) against the Greengenes database (gg\_13\_5) using `pick_closed_reference_otus.py` script, and then the PICRUSt pipeline<sup>[33]</sup> was used to predict bacterial functions. After using the `norma-lize_by_copy_number.py` script, the `predict_metagenomes.py` script was used to generate Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog predictions. We also applied the LEfSe method to find functional biomarkers in different groups.

### Statistical analysis

Data are presented as mean ± SD. The differences in data were statistically analyzed by one-way analysis of variance with Bonferroni's multiple-comparison test as *post hoc* analysis. SPSS version 20.0 was used for statistical analysis, and *P* < 0.05 was considered statistically significant.

## RESULTS

### Effects of Si-Ni-San freeze-dried powder on body weight, liver index and visceral fat index

At the beginning of the experiment, the body weight of the three groups of mice did not differ significantly (data not shown). After 12 wk, the FAT and SNS groups exhibited higher body weight, liver index and visceral fat index compared with the CON group (Figure 1A-C, *P* < 0.05, respectively). However, the SNS group, compared with the FAT group, showed decreased body weight, liver index and visceral fat index (Figure 1A-C, *P* < 0.05, respectively).

### Si-Ni-San freeze-dried powder ameliorates high fat diet-induced inflammation and liver injury

After 12 wk of eating the high fat diet, the FAT group had significantly increased levels of portal LPS, liver TNF- $\alpha$  and ALT compared with the CON group (Figure 2A-C, *P* < 0.05, respectively). However, compared with the FAT group, the SNS group, which was intragastrically administered Si-Ni-San freeze-dried powder every day for 12 wk, significantly decreased levels of portal LPS, liver TNF- $\alpha$  and ALT (Figure 2A-C, *P* < 0.05, respectively). Compared with the CON group, the SNS group only exhibited higher levels of liver TNF- $\alpha$  and ALT (Figure 2B and C, *P* < 0.05, respectively).

### Si-Ni-San freeze-dried powder reduces high fat diet-induced triglyceride accumulation in the liver

Oil Red O staining was used to morphologically observe triglyceride accumulation in the livers of mice. The CON group nearly had no triglyceride accumulation in the liver; however, the FAT group had obvious triglyceride accumulation (Figure 3A). The SNS group exhibited less triglyceride accumulation compared with the FAT group (Figure 3A). Triglyceride accumulation was measured using a colorimetric

**Table 2 Composition of diets**

	Normal chow diet in g/kg	High fat diet in g/kg
Casein	212.33	261.02
L-Cystine	2.84	3.50
Corn starch	275.84	56.87
Maltodextrin	33.18	116.53
Sucrose	331.77	201.36
Cellulose	47.40	58.26
Soybean oil	23.70	29.13
Lard	18.96	206.84
Mineral mix	9.84	11.65
Dicalcium phosphate	12.32	15.15
Calcium carbonate	5.21	6.41
Potassium citrate	15.64	19.23
Vitamin mix	9.48	11.56
Choline bitartrate	1.90	2.33
FD&C dye	0.047	0.058

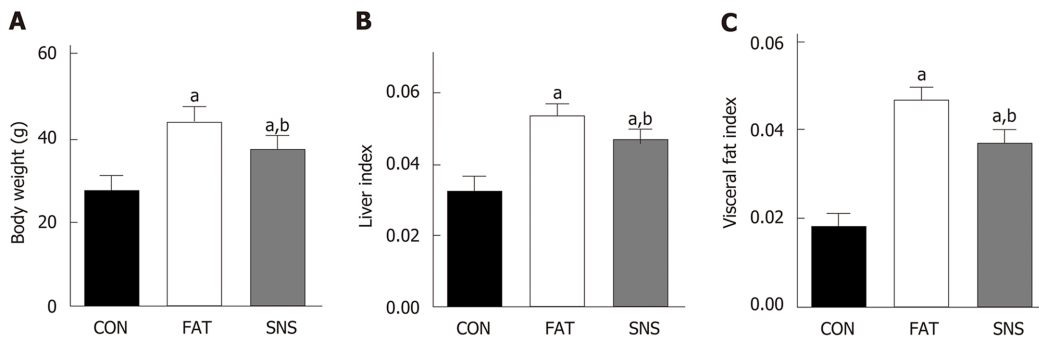
method, and the results were consistent with the findings of Oil Red O staining. The levels of triglycerides were significantly increased in the liver of the FAT and SNS groups compared with the CON group (Figure 3B,  $P < 0.05$ ). Si-Ni-San freeze-dried powder significantly decreased the levels of triglycerides in the SNS group compared with the FAT group (Figure 3B,  $P < 0.05$ ).

#### **Intestinal microbiota composition in different group samples**

A total of 1,380,651 high-quality reads (average of  $57,527 \pm 14,266$  sequences per sample) were used for biological information analysis, and 15,253 OTUs were clustered at 97% similarity (Figure 4A). Good coverage, which was  $> 0.96$ , and the rarefaction curves of all samples indicated that we obtained sufficient OTUs to reflect the bacterial composition of different samples accurately (Figure 4B). Although we calculated the values of Shannon Index and Chao 1, the three groups of mice showed no significant differences (data not shown). To observe the  $\beta$  diversity of different groups of mice, we used Unweighted UniFrac PCoA, and the result of this evolutionary distance-based method showed that the intestinal microbiota of the CON group was obviously separated from that of the FAT and SNS groups (Figure 4C). Despite partially overlapping, the intestinal microbiota of the FAT and SNS groups were roughly separated (Figure 4C). Analysis of molecular variance was used to further assess the spatial separation, and revealed that samples from the three groups differed significantly (CON *vs* FAT group,  $P < 0.001$ ; FAT *vs* SNS group,  $P < 0.001$ ; CON *vs* SNS group,  $P < 0.001$ ).

At the phylum level, 27 bacterial phyla were identified, and the top five were Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres and Actinobacteria. The phyla of BRC1, Chlamydiae, Chlorobi, Fibrobacteres, GN04 and OP3 were only found in the CON and SNS groups. Moreover, the bacterial phyla of Synergistetes and TM6 were only found in the SNS group. The FAT group samples increased the ratio of Firmicutes to Bacteroidetes compared with the CON group samples; however, this ratio in the SNS group samples did not differ significantly compared with the CON and FAT group samples. At the genus level, 227 genera were identified. To find taxon differences, we used the LEfSe method and showed that S24-7 (family level), Bacteroidales (order level), Bacteroidia (class level), Bacteroidetes (phylum level), *Mucispirillum* (genus level), Deferribacteraceae (family level), Deferribacterales (order level), Deferribacteres (class level), Deferribacteres (phylum level), Erysipelotrichaceae (family level), Erysipelotrichales (order level), Erysipelotrichi (class level), Desulfovibrionaceae (family level), Desulfovibrionales (order level), Delta-proteobacteria (class level), and Proteobacteria (phylum level) were biomarkers in the CON group samples (Figure 5A and B). *Lactobacillus* (genus level), Lactobacillaceae (family level), *Lactococcus* (genus level), Streptococcaceae (family level), Lactobacillales (order level), Bacilli (class level), and Firmicutes (phylum level) were biomarkers in the FAT group samples (Figure 5A and B). *Oscillospira* (genus level), Ruminococcaceae (family level), Clostridiales (order level), and Clostridia (class level) were biomarkers in the SNS group samples (Figure 5A and B).





**Figure 1** Effects of Si-Ni-San freeze-dried powder on body weight, liver index and visceral fat index. A: Body weight at 12 wk; B: Liver index (liver weight/body weight); C: Visceral fat index (visceral fat weight/body weight). <sup>a</sup> $P < 0.05$  vs CON group mice, <sup>b</sup> $P < 0.05$  vs FAT group mice.

### Intestinal microbiota functions in different group samples

We used PICRUST to predict the functions of bacterial microbiota using KEGG database at level 3, and then the LEfSe method to find functional biomarkers in different group samples. LEfSe showed that metabolism, genetic information processing, glycan biosynthesis and metabolism, energy metabolism, translation, amino acid metabolism, and replication and repair were functional biomarkers of the CON group samples (Figure 6A and B). Transcription and carbohydrate metabolism were functional biomarkers of the SNS group samples (Figure 6A and B). Environmental information processing, transporters, ABC transporters and transcription factors were functional biomarkers of the FAT group samples (Figure 6A and B).

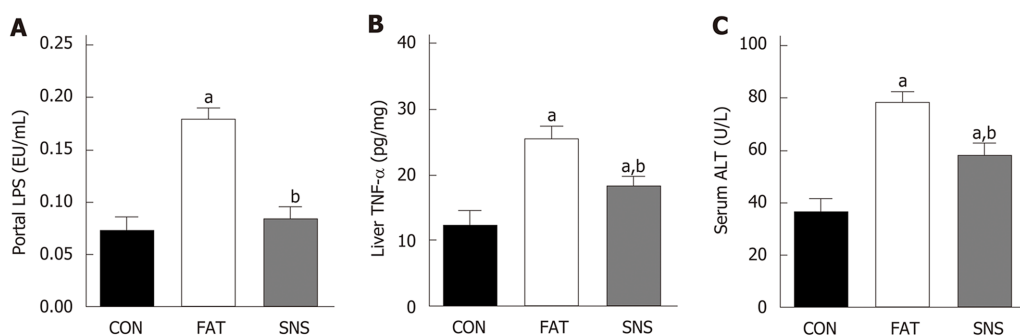
## DISCUSSION

The present study preliminarily investigated the effects of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD in mice. We demonstrated that Si-Ni-San freeze-dried powder ameliorates high fat diet-induced high levels of body weight, liver index, visceral fat index, portal LPS, serum ALT, liver TNF- $\alpha$  and liver triglyceride accumulation in mice. We also found that Si-Ni-San freeze-dried powder can alter intestinal microbiota composition and function in mice.

Previous research has studied the effects of Si-Ni-San on NAFLD, and found that Si-Ni-San can reduce the liver concentration of total cholesterol, triglyceride, free fatty acid and interleukin (IL)-6 in rats with NAFLD<sup>[34]</sup>. However, animal models of NAFLD, in this research, were induced using a long-term chronic stress method that is not widely used in NAFLD studies. In addition, this method caused a decrease in body weight compared with control rats, which does not match the real-life situation. In the present study, we adopted a high fat diet to induce animal models of NAFLD, which is a stable and widely used method in NAFLD studies. This method always induces obesity, insulin resistance, dyslipidemia and liver triglyceride accumulation, which more accurately reflect real-life situations. Thus, our present study has a wider practical significance.

NAFLD is always considered to be the hepatic manifestation of metabolic syndrome, and insulin resistance plays an important role in the pathogenesis of NAFLD<sup>[35-37]</sup>. In the present study, although the level of insulin resistance in the SNS group exhibited a decreased trend compared with the FAT group, the difference was not significant (data not shown), suggesting that improving insulin resistance is not a mechanism of action of Si-Ni-San freeze-dried powder, or its improvement of insulin resistance is not due to small sample size. Thus, additional large-sample studies addressing the effect of Si-Ni-San freeze-dried powder on insulin resistance are warranted.

Previous studies have revealed the key role of LPS, a constituent of Gram-negative bacteria and main constituent of endotoxemia, in the development of metabolic diseases, and elevated LPS has been observed in patients with NAFLD<sup>[25,38]</sup>. LPS is an inflammatory trigger, and it can promote the secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , IL-1 and IL-6. In the present study, we found that high fat diet increased the levels of portal LPS and liver TNF- $\alpha$  compared with normal chow diet; however, Si-Ni-San freeze-dried powder reduced these increases,



**Figure 2 Si-Ni-San freeze-dried powder ameliorates liver inflammation and injury.** A: Portal lipopolysaccharide; B: Liver tumor necrosis factor- $\alpha$ ; C: Serum alanine aminotransferase. <sup>a</sup> $P < 0.05$  vs CON group mice, <sup>b</sup> $P < 0.05$  vs FAT group mice. ALT: Alanine aminotransferase; LPS: Lipopolysaccharide; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

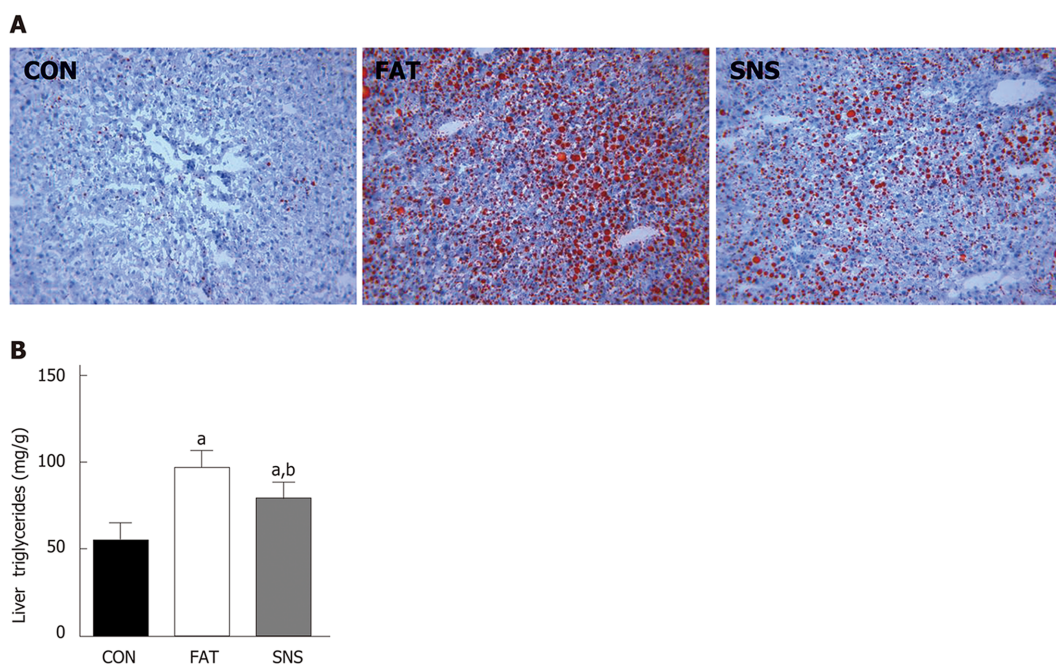
suggesting that the anti-inflammatory effect of Si-Ni-San freeze-dried powder may be a mechanism underlying its beneficial effect. Although the portal LPS of the CON and SNS groups did not differ significantly, the SNS group only exhibited higher levels of liver TNF- $\alpha$  and ALT, suggesting that in addition to LPS, there may be other proinflammatory factors.

The important role of intestinal microbiota in the pathogenesis of NAFLD has been revealed in previous studies<sup>[22,39]</sup>. Given the tight links between intestinal microbiota and NAFLD, manipulation of the intestinal microbiota of NAFLD is a promising therapeutic strategy<sup>[40-42]</sup>. Thus, in the present study, we investigated the effect of Si-Ni-San freeze-dried powder on intestinal microbiota, and found that the intestinal microbiota composition and function in the SNS group differed clearly from those in the FAT group, suggesting that Si-Ni-San freeze-dried powder alters high fat diet-induced intestinal microbiota dysbiosis. So, the therapeutic effect of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD may be achieved by changing the intestinal microbiota. The fact that the *Oscillospira* genus was a bacterial biomarker in the SNS group samples demands our further attention. Up to now, there has been little knowledge about this genus, mainly because it has never been cultured. Our former study found that *Oscillospira* is associated with a high protein diet, which has beneficial effects on NAFLD<sup>[30]</sup>. Other studies have found that *Oscillospira* is significantly reduced in patients with NASH or inflammatory bowel disease<sup>[43-45]</sup>, and is positively associated with leanness<sup>[46]</sup>. A recent study has found that some *Oscillospira* species may be able to utilize glycans to secrete butyrate<sup>[47]</sup>, which can prevent inflammation<sup>[48]</sup>, and plays an important role in metabolic diseases<sup>[49-51]</sup>. Using a combination of our results with those of former studies, we infer that Si-Ni-San freeze-dried powder may achieve its beneficial effect by providing more special glycans to *Oscillospira* bacteria and then producing more butyrate, which has multiple beneficial effects on host health. The result that carbohydrate metabolism was a functional biomarker in the SNS group partly supports our inference, and further studies are warranted to confirm our hypothesis.

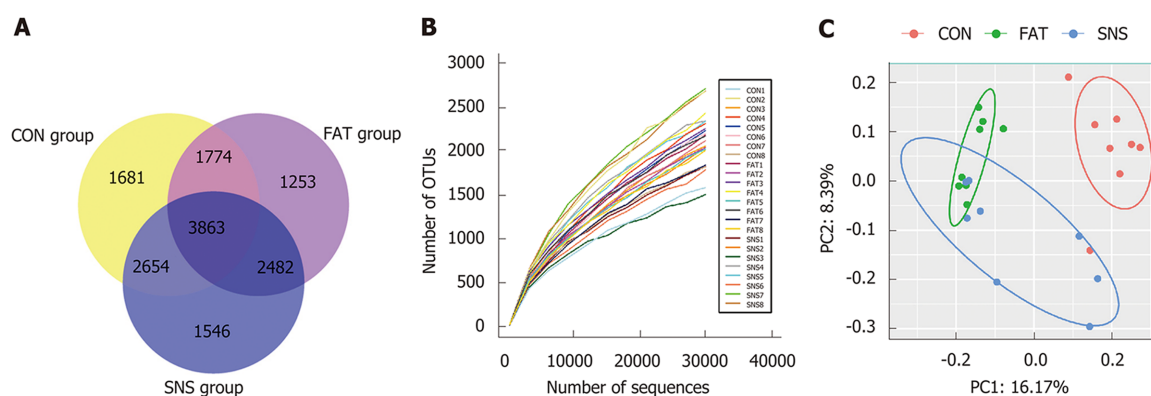
An interesting finding in our study was that *Lactobacillus* genus was a bacterial biomarker of the FAT group. Some strains of *Lactobacillus* are frequently used as probiotics<sup>[52]</sup>. Thus, our finding needs confirmation by further studies.

There were several limitations to the present study. First, there was a lack of metabolomics analysis, so the effects of Si-Ni-San freeze-dried powder on the metabolism of intestinal microbiota are not known. Second, we did not administer Si-Ni-San freeze-dried powder to normal chow diet mice, so the effects of Si-Ni-San freeze-dried powder on normal chow diet mice are not known. Third, the sample size was small, so our results need further studies for confirmation. Fourth, the change in some cytokines, such as IL-1 and IL-6, was not detected in the present study. These limitations will be addressed in our future studies.

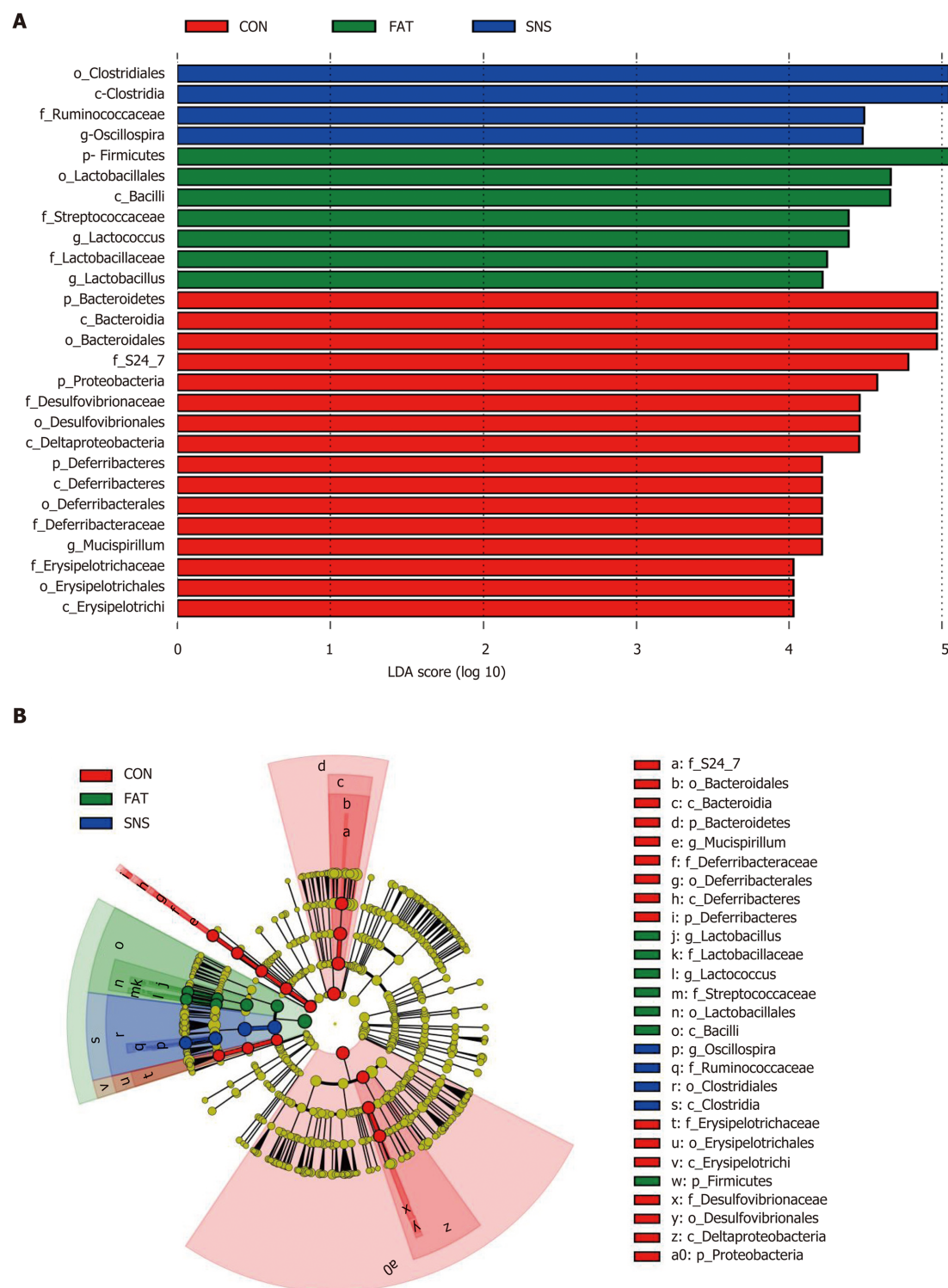
In conclusion, our present study preliminarily confirmed the beneficial effects of Si-Ni-San freeze-dried powder on high fat diet-induced NAFLD in mice, and that the mechanisms of action of Si-Ni-San freeze-dried powder against NAFLD may be associated with its anti-inflammatory effects and its changes to the intestinal microbiota. Our findings provide some useful information for NAFLD therapy. We provide the basis for the clinical use of Si-Ni-San freeze-dried powder and some underlying mechanisms of its action. Although more in-depth research is needed in the future, Si-Ni-San freeze-dried powder may also be a clinical option for NAFLD treatment.



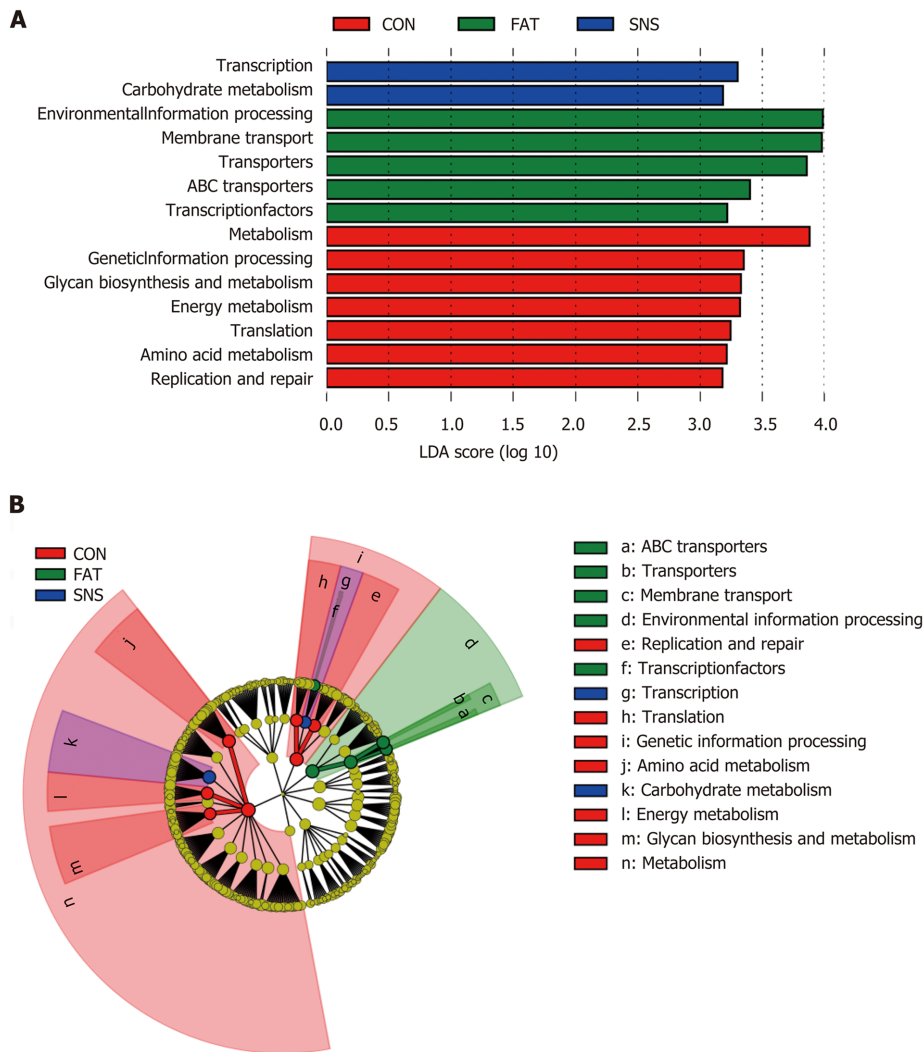
**Figure 3** Si-Ni-San freeze-dried powder ameliorates high fat diet-induced hepatic triglyceride accumulation. A: Representative Oil Red O staining (magnification 200 ×); B: Liver triglycerides. <sup>a</sup> $P < 0.05$  vs CON group mice, <sup>b</sup> $P < 0.05$  vs FAT group mice.



**Figure 4** Intestinal microbiota composition in different group samples. A: Venn diagram of operational taxonomic units; B: Rarefaction curves; C: Unweighted UniFrac PCoA. OTUs: Operational taxonomic units.



**Figure 5 Bacterial biomarkers of different group samples.** A: Bacterial biomarkers found by linear discriminant analysis effect size (LEfSe); B: Taxonomic cladogram obtained from LEfSe. Taxa meeting an linear discriminant analysis significant threshold > 4 are shown. c: class level; f: family level; g: genus level; o: order level; p: phylum level. LDA: Linear discriminant analysis.



**Figure 6** Intestinal microbiota functions in different group samples. A: Functional biomarkers found by linear discriminant analysis effect size (LEfSe); B: Functional cladogram obtained from LEfSe. Functions meeting an linear discriminant analysis significant threshold  $> 3.1$  are shown. LDA: Linear discriminant analysis.

## ARTICLE HIGHLIGHTS

### Research background

The incidence of nonalcoholic fatty liver disease (NAFLD) dramatically increased in the last few decades. Unfortunately, until now, the clinical treatment of this common chronic liver disease is difficult, and some new effective therapies are needed.

### Research motivation

Some herbal medicines have hepatoprotective effects, so we want to know if some famous prescriptions of traditional Chinese medicine can provide beneficial effects on NAFLD.

### Research objectives

To explore the effects of Si-Ni-San, a famous prescription of traditional Chinese medicine, on NAFLD and intestinal microbiota.

### Research methods

We intragastrically administered Si-Ni-San freeze-dried powder (5.0 g/kg) to mice, which were allowed ad libitum access to a high fat diet. After 12 wk of treatment, we measured body weight, liver index, visceral fat index, serum alanine aminotransferase (ALT), portal lipopolysaccharide (LPS), liver tumor necrosis factor (TNF)- $\alpha$ , liver triglycerides and intestinal microbiota, and we compared the results of these parameters with mice in another group to find whether Si-Ni-San freeze-dried powder have some beneficial effects on NAFLD.

### Research results

After Si-Ni-San freeze-dried powder treatment, the levels of body weight, liver index, visceral fat



index, serum ALT, portal LPS, liver TNF- $\alpha$  and liver triglycerides were improved. The composition of intestinal microbiota was also changed, especially the *Oscillospira* genus.

### Research conclusions

Si-Ni-San freeze-dried powder can ameliorate NAFLD by an anti-inflammatory action and intestinal microbiota-changing effect.

### Research perspectives

Although we provide basis for the clinical use of Si-Ni-San freeze-dried powder and some underlying mechanisms of its action, the effects of Si-Ni-San freeze-dried powder on the metabolism of intestinal microbiota and some cytokines, such as interleukin (IL)-1 and IL-6, need to be addressed in future studies.

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## Retrospective Study

# Clinical characteristics of young patients with early Barrett's neoplasia

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## Abstract

### BACKGROUND

Esophageal adenocarcinoma (EAC) and high-grade dysplasia (HGD) may appear in young patients with Barrett's esophagus (BE). However, characteristics of Barrett's-related neoplasia in this younger population remain unknown.

### AIM

To identify clinical characteristics that differ between young and old patients with early-stage Barrett's-related neoplasia.

### METHODS

We conducted a retrospective analysis of a prospectively maintained database comprised of consecutive patients with early-stage EAC (pT1) and HGD at a tertiary-referral center between 2001 and 2017. Baseline characteristics, drug and risk factor exposures, clinicopathological staging of EAC/HGD and treatment outcomes [complete eradication of neoplasia (CE-N), complete eradication of intestinal metaplasia (CE-IM), recurrence of neoplasia and recurrence of intestinal metaplasia] were retrieved. Multivariate analyses were performed to identify factors that differed significantly between older and younger ( $\leq 50$  years) patients.

### RESULTS

We identified 450 patients with T1 EAC and HGD (74% and 26%, respectively); 45 (10%) were  $\leq 50$  years. Compared to the older group, young patients were

inclusion in our database were obtained from all participants.

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more likely to present with ongoing gastroesophageal reflux disease (GERD) symptoms (55% *vs* 38%,  $P = 0.04$ ) and to be obese (body mass index  $> 30$ , 48% *vs* 32%,  $P = 0.04$ ). Multivariate logistic regression analysis showed that young patients were significantly more likely to have ongoing GERD symptoms [odds ratio (OR) 2.00, 95% confidence interval (CI) 1.04-3.85,  $P = 0.04$ ] and to be obese (OR 2.06, 95%CI 1.07-3.98,  $P = 0.03$ ) whereas the young group was less likely to have a smoking history (OR 0.39, 95%CI 0.20-0.75,  $P < 0.01$ ) compared to the old group. However, there were no significant differences regarding tumor histology, CE-N, CE-IM, recurrence of neoplasia and recurrence of intestinal metaplasia (mean follow-up, 44.3 mo).

## CONCLUSION

While guidelines recommend BE screening in patients  $> 50$  years of age, younger patients should be considered for screening endoscopy if they suffer from obesity and GERD symptoms.

**Key words:** Barrett's Esophagus; Gastroesophageal reflux disease; Obesity; Esophageal adenocarcinoma; High-grade dysplasia; Guideline; Young patient

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**Core tip:** Esophageal adenocarcinoma (EAC) and high-grade dysplasia (HGD) may appear in young patients with Barrett's esophagus (BE). To identify clinical characteristics of young patients with Barrett's neoplasia, we conducted a retrospective analysis. 450 patients with T1 EAC and HGD were identified; 45 (10%) were young patients at age  $\leq 50$  years. Compared to the older group, young patients were more likely to present with ongoing gastroesophageal reflux disease (GERD) symptoms and to be obese on multivariate analysis. While guidelines recommend BE screening in patients  $> 50$  years of age, younger patients should be considered for screening endoscopy if they suffer from obesity and GERD symptoms.

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## INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition for esophageal adenocarcinoma (EAC)<sup>[1]</sup>, which has a poor prognosis with a 5-year survival rate below 20%<sup>[2]</sup>. However, screening endoscopy of the general population for BE or EAC is not recommended because of the low incidence of EAC and the lack of randomized controlled trials supporting its efficiency<sup>[3-6]</sup>. According to recent guidelines, screening should be considered only in patients with multiple risk factors for BE or EAC; such as long standing gastroesophageal reflux disease (GERD) symptoms, age  $> 50$  years, white race, male sex, obesity, family history of BE or EAC, and smoking<sup>[3-6]</sup>.

Older age is one of the most important risk factors for BE<sup>[7,8]</sup>. Most guidelines set the cut-off at age 50. On the other hand, the diagnosis of Barrett's-related neoplasia in younger patients is becoming more common in daily clinical practice. In fact, the incidence rate of EAC for the young has been steadily increasing in recent years<sup>[9]</sup>. However, the clinical characteristics of these younger EAC patients are poorly known. Even though some studies have evaluated the prognosis of EAC among young patients<sup>[10-12]</sup>, few articles have identified the baseline clinical characteristics of this patient group with EAC<sup>[13,14]</sup>. Moreover, features and outcomes of young patients with early-stage EAC are poorly described. Yet detecting early-stage neoplasia holds the opportunity for curative endoscopic resection with excellent long-term outcomes. Thus, if this younger cohort differs significantly with respect to specific clinical characteristics from the more typical age category of BE neoplasia, these features could help to improve screening recommendations. Hence, we conducted a retrospective analysis of a prospectively maintained database of patients diagnosed



with BE and early-stage EAC/high-grade dysplasia (HGD) to identify factors associated with the development of Barrett's-related neoplasia occurring in younger patients. Additionally, we examined for any correlation between age groups and treatment outcomes.

## MATERIALS AND METHODS

### *Study participants*

We conducted a retrospective analysis of a prospective database comprised of consecutive patients with early-stage EAC (T1a and T1b) and HGD in BE at a single, tertiary-referral center (St Michael's Hospital, Toronto, Canada) between May 2001 and May 2017. For cases that occurred prior the establishment of the Prague criteria, we only included cases in which the circumferential and maximum BE length were documented at their index endoscopy<sup>[15]</sup>. Exclusion criteria were: (1) Patients who did not undergo endoscopic mucosal resection (EMR) (because of the absence of precise histopathological staging); (2) Patients who underwent esophagectomy or chemo/radiotherapy due to unfavorable features of the first EMR specimen such as submucosal invasion, poorly differentiated cancer (G3) and lympho-vascular invasion (Figure 1). All patients provided written informed consent for their inclusion in our database. The study was carried out in accordance with the Declaration of Helsinki and was approved by the St. Michael's Hospital research ethics committee (#08-265).

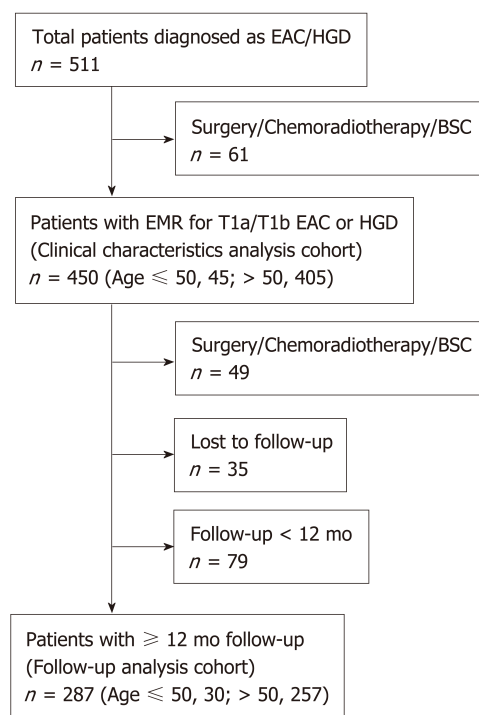
### *Data collection and procedures*

All patients provided detailed information via a demographic, medical history, and lifestyle questionnaire that ascertained the following characteristics: age at diagnosis for EAC or HGD, sex, ethnicity, height, weight, body mass index (BMI), comorbidities, family history of malignancy including EAC, tobacco and alcohol consumption, ongoing GERD-related symptoms (defined as having at least two episodes of reflux symptoms within the most recent three months) and medication [including proton pump inhibitors (PPIs), low-dose aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and statins].

Regarding endoscopic findings, the circumferential (C) and maximum (M) length of BE based on the Prague criteria were systematically recorded<sup>[15]</sup>. The recorded distance from the diaphragm to the gastroesophageal junction, defined as the oral end of the gastric folds, was used to determine the presence or absence of a hiatus hernia. According to the Seattle protocol, multiple, 4-quadrant biopsies were obtained every 1 to 2 cm to identify intestinal metaplasia or dysplasia. EMR was performed when a visible lesion was found or to completely eradicate BE via radical EMR, particularly for short-segment BE. Most patients underwent radiofrequency ablation (RFA) of the remaining BE segments for complete eradication of BE, as described elsewhere<sup>[16]</sup>. Some patients underwent other eradication techniques such as bipolar electrocoagulation (BiCAP), photodynamic therapy (PDT), cryotherapy and hot avulsion due to the evolution of treatment strategies during the study period. Hot avulsion is our previously described technique used to eradicate small persistent BE areas 1 cm or less using hot biopsy forceps with cauterization<sup>[17]</sup>.

All specimens were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin staining. At least two experienced gastrointestinal pathologists analyzed all specimens. A diagnosis of BE was made based on the presence of intestinal metaplasia with goblet cells. The final T-staging diagnosis was based on pathology from the EMR specimen. Pathological reports were recorded in accordance with the Vienna classification<sup>[18]</sup> and EMR reports included the information such as grade of differentiation (G), depth of invasion according to the Vieth and Stolte system (M1-4, SM)<sup>[19]</sup>, vertical margin and the presence or absence of lymphovascular invasion (LVI).

For assessment of endoscopic treatment-related outcomes, we included only patients followed up for more than 12 mo after initial EMR. Patients who underwent EMR were followed-up by endoscopy in 3- to 6-mo intervals with surveillance biopsies and additional endoscopic therapies (ablative methods or additional EMR), at the discretion of the endoscopist, until all visible BE was eradicated. Complete eradication of neoplasia (CE-N) was defined as the absence of endoscopic and pathologic evidence of adenocarcinoma or any dysplasia after endoscopic treatment. Complete eradication of intestinal metaplasia (CE-IM) was defined as complete absence of endoscopic evidence of BE and pathologic evidence of intestinal metaplasia on all follow-up biopsies. Recurrence of neoplasia and intestinal metaplasia were defined as any biopsy-confirmed dysplastic lesion and intestinal metaplasia detected on subsequent endoscopies following CE-N and CE-IM, respectively.



**Figure 1** Flow diagram for the study patients. EAC: Esophageal adenocarcinoma; HGD: High-grade dysplasia; BSC: Best supportive care; EMR: Endoscopic mucosal resection.

### Statistical analysis

The mean  $\pm$  standard deviation (SD) was used for variables with a normal distribution, and the median and interquartile range was used for variables with a skewed distribution. Differences between groups were analyzed using the Chi-square test and Fisher's exact for categorical data, the Student *t*-test for comparing means and the Mann-Whitney *U* test for comparing medians for continuous data. Multivariable logistic regression models were performed to identify clinical and pathologic differences between younger ( $\leq 50$  years) and older patients. We included the following variables in the multivariate logistic regression analysis: BMI ( $> 30$  or  $< 30$ ), history of smoking, ongoing GERD symptoms, family history of EAC, ethnicity (white) and sex (male); as these factors are well known to be associated with EAC and used to guide screening endoscopy in most guidelines<sup>[3-6]</sup>. We calculated adjusted odds ratios (ORs) and 95% confidence intervals (CIs) by multivariate logistic regression analysis using StatFlex software (Artech Co., Osaka, Japan). Two-sided *P* values  $< 0.05$  were considered statistically significant.

## RESULTS

### Study population and clinical characteristics

We identified 450 patients diagnosed with Barrett's-related EAC (T1a or T1b) or HGD during the study period. Of these, 45 patients (10%) were 50 years of age or younger [39 men (87%)] and 405 patients (90%)  $> 50$  years [342 men (84%)].

Patient clinical characteristics are summarized in [Table 1](#). Male and white patients were predominant in both groups with no significant differences. Young patients were more likely to be obese (BMI  $> 30$ , 48% *vs* 32%, *P* = 0.04) and to have ongoing GERD symptoms (55% *vs* 38%, *P* = 0.04), and less likely to have diabetes and hypertension, and to have ever been smokers on univariate analysis. There were no significant differences between the groups with regards to family history of EAC and alcohol consumption. Regarding medication use, we found that the older group was more likely to have used low-dose aspirin and statins compared to the younger patients, while there were no significant differences regarding use of PPIs and NSAIDs. With regard to endoscopic findings, the median circumferential extent of BE (Prague C) in the young and old groups were 2 cm and 1cm, respectively (*P* = 0.52) and the median maximal extent of BE (Prague M) was 4 cm and 4cm, respectively (*P* = 0.43). The prevalence of a hiatus hernia was not significantly different between groups

( $P = 0.12$ ).

Multivariate modeling (Table 2) showed that young patients were significantly more likely to be obese (BMI > 30, OR 2.06, 95%CI 1.07-3.98,  $P = 0.03$ ) and to have ongoing GERD symptoms (OR 2.00, 95%CI 1.04-3.85,  $P = 0.04$ ), whereas the young group was less likely to have a smoking history (OR 0.39, 95%CI 0.20-0.75,  $P < 0.01$ ) compared to the old group.

### Pathological features

A comparison of the pathological features between young and old patients is summarized in Table 3. Thirty-one (69%) and 317 (78%) patients had EAC among young and old groups, respectively ( $P = 0.15$ ). There were no significant differences between the groups in terms of depth of EAC invasion, tumor differentiation, LVI and rate of positive vertical (deep) margin.

### Endoscopic treatment-related outcomes and follow-up

Clinical outcomes following endoscopic treatment were available for 287 patients who met inclusion criteria (Figure 1, young,  $n = 30$ ; old,  $n = 257$ ). Mean follow-up duration was  $44.3 \pm \text{SD: } 30.2$  mo. All patients underwent EMR and 176 patients had additional one or more ablative therapies following the first EMR; RFA ( $n = 114$ ), BiCAP ( $n = 17$ ), PDT ( $n = 14$ ), cryotherapy ( $n = 3$ ) and hot avulsion ( $n = 107$ ). The overall rates of CE-N and CE-IM were 86% and 63%, respectively. There were no significant differences between young and old groups in terms of CE-N (93% *vs* 86%,  $P = 0.38$ ) and recurrence rates of neoplasia after CE-N (14.3% *vs* 18%,  $P = 0.81$ ) (Table 4). Similarly, no differences were found regarding CE-IM (77% *vs* 62%,  $P = 0.16$ ) and recurrence rates of intestinal metaplasia after CE-IM (30% *vs* 26%,  $P = 0.83$ ).

## DISCUSSION

The clinical and pathologic characteristics of early-stage EAC in young patients has been poorly documented because of its low incidence. Our current study, based on a large prospective cohort of patients diagnosed with early-stage BE neoplasia, suggests that younger patients ( $\leq 50$  years) with early-stage EAC or HGD were more likely to have ongoing GERD symptoms and to be obese compared to their older counterparts. Furthermore, we found that there were no significant differences in terms of endoscopic treatment-related outcomes between groups.

The incidence of EAC in young patients has been generally considered to be very low. Consequently, most guidelines recommend screening endoscopy only for patients > 50 years who have multiple risk factors. Murphy *et al*<sup>[9]</sup> demonstrated that the incidence of EAC amongst younger patients has been increasing just as the total number of EAC patients has increased. Thrift *et al*<sup>[20]</sup> speculated that declining infection rates of *Helicobacter pylori* may lead to higher rates of EAC in young cohorts in the near future, given the theory that this infection may reduce the risk for EAC. Indeed, our data revealed that the percentage of young early-stage EAC/HGD patients was significant (45/450, 10%). Another recent study using a large cohort demonstrated that the proportion of young ( $\leq 50$  years) EAC patients was 9% (125/1363)<sup>[14]</sup> which is similar to our results, underlining that a significant number of Barrett's-related neoplasms appear before the age of 50.

The prognosis of young EAC patients is still controversial. Some studies have reported that young patients with EAC presented with a more advanced stage of the disease and had poorer survival than older EAC patients<sup>[11,12,21]</sup>. In our cohort, the outcomes of endoscopic treatment for T1 EAC or HGD between both groups were similar. Therefore, it becomes of utmost importance to elucidate the risk factors that may facilitate the early detection of EAC in this younger population.

GERD symptoms are the most important risk factor for BE and EAC<sup>[3,5,6]</sup>. Cook *et al*<sup>[22]</sup>, using a large pooled analysis of five population-based case-control studies, showed that the risk of EAC in patients with GERD symptoms for at least 30 years was 6.2-fold higher than in patients without GERD symptoms. On the other hand, another analysis comprising three additional studies revealed that this association was stronger in patients under the age of 50<sup>[14]</sup>. Similarly, we found that 92% of younger patients had ongoing GERD symptoms despite taking PPIs in our cohort (data not shown), which suggests that refractory GERD is strongly related to EAC risk in younger patients. However, Becher *et al*<sup>[23]</sup> demonstrated that GERD symptoms are more severe among younger than older patients, while aging is associated with more severe patterns of acid reflux and reflux esophagitis. Therefore, the mere presence of GERD symptoms is not sufficient to perform endoscopy. Additional risk factors should be considered in order to increase the efficiency of screening endoscopy especially among young patients.

Table 1 Patient characteristics

	Young ( $\leq 50$ yr)	Old ( $> 50$ yr)	P value
	(n = 45)	(n = 405)	
Sex (male)	39/45 (87%)	342/405 (84%)	0.86
Ethnicity (white)	43/44 (98%)	377/387 (97%)	0.70
BMI, kg/m <sup>2</sup> (mean $\pm$ SD)	29.69 $\pm$ 6.05	28.53 $\pm$ 5.72	0.09
BMI $> 30$ kg/m <sup>2</sup>	21/44 (48%)	124/389 (32%)	0.04
Diabetes	5/45 (11%)	91/390 (23%)	0.01
Hypertension	13/45 (29%)	211/382 (55%)	$< 0.001$
Family history of malignancy	21/44 (48%)	234/382 (61%)	0.08
Family history of esophageal adenocarcinoma	1/44 (2%)	17/382 (5%)	0.78
GERD symptoms	24/44 (55%)	148/386 (38%)	0.04
Smoking			
Ever smoking	25/44 (57%)	297/393 (76%)	0.01
Current smoking	4/44 (9%)	47/392 (11%)	0.75
Pack-years (mean $\pm$ SD)	13.97 $\pm$ 15.56	23.46 $\pm$ 27.21	0.02
Alcohol			
Ever alcohol	37/45 (82%)	297/387 (77%)	0.52
Current alcohol	31/45 (69%)	241/375 (64%)	0.54
Medication use			
Proton pump inhibitors	38/43 (88%)	335/374 (90%)	0.98
Low-dose aspirin	5/45 (11%)	130/388 (34%)	$< 0.01$
NSAIDs	4/45 (9%)	34/384 (9%)	0.79
Statins	10/45 (22%)	189/388 (49%)	$< 0.001$
Endoscopy			
Prague C, median (IQR), cm	2 (0-5)	1 (0-5)	0.52
Prague M, median (IQR), cm	4 (2-7)	4 (2-7)	0.43
Hiatus hernia	35/45 (78%)	350/405 (86%)	0.12

BMI: Body mass index; GERD: Gastroesophageal reflux disease; NSAIDs: Nonsteroidal anti-inflammatory drugs; SD: Standard deviation; IQR: Interquartile range.

Obesity is another important risk factor for BE and EAC<sup>[24]</sup>. Central adiposity is thought to be more critical for esophageal carcinogenesis than merely increased BMI, since central adiposity contributes not only to increasing acid reflux due to mechanical disruption of the gastroesophageal junction, but also promotes a proinflammatory state by releasing adipocytokines from visceral adipose tissue<sup>[25]</sup>. Adipocytokine-mediated carcinogenesis is thought to play an important role in other gastrointestinal malignancies such as colon<sup>[26]</sup> and pancreas<sup>[27]</sup> cancers. However, few studies have investigated the relationship between obesity and gastrointestinal carcinogenesis specifically in young patients. How obesity and central adiposity affect EAC risk, especially in young patients, remains unclear. Chak *et al*<sup>[13]</sup> demonstrated that obesity is associated with the development of EAC at an earlier age. We also found that obesity was more common in young patients with early-stage Barrett-related neoplasia. Altogether, our results suggest that screening endoscopy should be strongly considered in obese young patients with ongoing GERD symptoms.

Additionally, our results showed that any history of smoking had a stronger association with the older EAC/HGD group ( $> 50$  years). Although cigarette smoking is a well-known risk factor for the development of both BE and EAC<sup>[28,29]</sup>, the precise role of smoking in EAC carcinogenesis remains unclear. We have to emphasize that this result does not suggest smoking has a protective role for Barrett's carcinogenesis in young cohort. In our cohort, the number of pack-years in older EAC group was significantly higher. We hypothesized that this association could be related to the accumulation of toxicity for a longer time span. We also thought why our results showed older patients was more likely to have hypertension and diabetes, and to take low-dose aspirin and statins were just due to aging.

It should be highlighted that our study population was based exclusively on patients diagnosed with early stage neoplasia. To the best of our knowledge, this is the first study to compare the baseline clinical characteristics and treatment outcomes

**Table 2** Adenocarcinoma/high-grade dysplasia risk according to multivariate analysis: Young vs old

	Adjusted Odds ratio	95% confidence interval	P value
BMI > 30	2.06	1.07-3.98	<b>0.03</b>
Ever smoking	0.39	0.20-0.75	<b>&lt; 0.01</b>
Ongoing GERD symptoms	2.00	1.04-3.85	<b>0.04</b>
Family history of esophageal adenocarcinoma	0.73	0.09-5.85	0.77
Ethnicity (white)	0.77	0.09-6.42	0.81
Sex (Male)	1.44	0.56-3.70	0.45

BMI: Body mass index; GERD: Gastroesophageal reflux disease.

between the young and old onset groups using a relatively large cohort including only early stage Barrett's-related neoplasia. On the other hand, including only HGD and T1 tumor might be also interpreted as a limitation. However, focusing on early Barrett's-related neoplasia amenable to endoscopic treatment allowed us to develop a suggestion that if we could detect the young patients with early stage Barrett's neoplasia using the risk factors of ongoing GERD symptoms and obesity, their prognosis may compare favorably with older patients. We believe that this can complement the current guidelines for Barrett's screening.

This study has some limitations. First, we did not have control groups such as young and old patients without Barrett's-related neoplasia. Because of this point and retrospective nature of our study, we cannot say that GERD symptoms and obesity are predictive factors for EAC in young patients. Therefore, the risk factors that we extracted should be proved by a prospectively designed study. Second, as our institution is a tertiary referral center, our cohort may not be representative of the general population or community practice BE population. Third, due to the retrospective nature of the study, some patients were lost to follow-up. Therefore, we could not assess long-term morbidity and mortality.

In conclusion, we identified that patients  $\leq 50$  years old with early-stage EAC or HGD had greater odds of having ongoing GERD symptoms and to be obese than older patients. Our results may serve to improve the selection of younger patients who would most benefit from screening endoscopy. Further prospective studies are needed to clarify the risk factors specific to young patients with Barrett's-related neoplasia.



**Table 3** Pathological features of esophageal adenocarcinoma, *n* (%)

		Young ( $\leq 50$ yr)	Old ( $> 50$ yr)	<i>P</i> value
		( <i>n</i> = 45)	( <i>n</i> = 405)	
Histology	EAC	31 (69)	317 (78)	0.15
	HGD	14 (31)	88 (22)	
EAC depth	M1	7 (23)	63 (20)	0.41
	M2	8 (26)	75 (24)	
	M3	8 (26)	47 (15)	
	M4	4 (13)	96 (30)	
	SM	4 (13)	36 (11)	
	G1	21 (68)	212 (67)	
Differentiation	G2	6 (19)	91 (29)	0.22
	G3	4 (13)	14 (4)	
Lympho-vascular invasion		1 (3)	32 (10)	0.36
Vertical margin positive rate		3 (10)	32 (10)	0.81

EAC: Esophageal adenocarcinoma; HGD: High-grade dysplasia; M: Mucosa; SM: Submucosa.

**Table 4** Clinical outcomes following endoscopic treatment

	Young ( $\leq 50$ yr)	Old ( $> 50$ yr)	<i>P</i> value
	( <i>n</i> = 30)	( <i>n</i> = 257)	
Complete eradication of neoplasia	28/30 (93%)	220/257 (86%)	0.38
Recurrence of neoplasia	4/28 (14%)	40/220 (18%)	0.81
Complete eradication of intestinal metaplasia	23/30 (77%)	159/257 (62%)	0.16
Recurrence of intestinal metaplasia	7/23 (30%)	41/159 (26%)	0.83

## ARTICLE HIGHLIGHTS

### Research background

Older age is one of the most important risk factors for Barrett's esophagus. Most guidelines set the cut-off at age 50. On the other hand, the diagnosis of Barrett's neoplasia in younger patients is becoming more common in daily clinical practice.

### Research motivation

The clinical characteristics of these younger esophageal adenocarcinoma (EAC) and high-grade dysplasia (HGD) patients are poorly known. If this younger cohort differs significantly with respect to specific clinical characteristics from the more typical age category of Barrett's neoplasia, these features could help to improve screening recommendations.

### Research objectives

To identify factors associated with the development of Barrett's neoplasia occurring in younger patients.

### Research methods

A retrospective analysis of a prospectively maintained database comprised of consecutive patients with early-stage EAC (pT1) and HGD at a tertiary-referral center between 2001 and 2017 was conducted. Baseline characteristics, drug and risk factor exposures, clinicopathological staging of EAC/HGD and treatment outcomes [complete eradication of neoplasia (CE-N), complete eradication of intestinal metaplasia (CE-IM), recurrence of neoplasia and recurrence of intestinal metaplasia] were retrieved. Multivariate analyses were performed to identify factors that differed significantly between older and younger ( $\leq 50$  years) patients.

### Research results

Four hundred fifty patients with T1 EAC and HGD were enrolled in this study. Forty-five patients (10%) were  $\leq 50$  years. Compared to the older group, young patients were more likely to have ongoing gastroesophageal reflux disease (GERD) symptoms and to be obese. The same pattern of differences was maintained with an even greater magnitude of effects on multivariate analysis. However, there were no significant differences regarding tumor histology, CE-N, CE-

IM, recurrence of neoplasia and recurrence of intestinal metaplasia (mean follow-up, 44.3 mo).

### Research conclusions

We identified that patients  $\leq 50$  years old with early-stage EAC or HGD had greater odds of having ongoing GERD symptoms and to be obese than older patients. Our results may serve to improve the selection of younger patients who would most benefit from screening endoscopy.

### Research perspectives

Further prospective studies are needed to clarify the risk factors specific to young patients with Barrett's-related neoplasia.

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## Observational Study

# Evaluation of clinical outcomes in an interdisciplinary abdominal pain clinic: A retrospective, exploratory review

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### Institutional review board

**statement:** This study was reviewed and approved by the Children's Mercy Hospital Pediatric Institutional Review Board.

### Informed consent statement:

Waivers of permission/assent and consent were deemed appropriate for this study.

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None to declare.

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## Abstract

### BACKGROUND

Pediatric functional gastrointestinal disorders (FGIDs) are common and well-accepted to be etiologically complex in terms of the contribution of biological, psychological, and social factors to symptom presentations. Nonetheless, despite its documented benefits, interdisciplinary treatment, designed to address all of these factors, for pediatric FGIDs remains rare. The current study hypothesized that the majority of pediatric patients seen in an interdisciplinary abdominal pain clinic (APC) would demonstrate clinical resolution of symptoms during the study period and that specific psychosocial variables would be significantly predictive of GI symptom improvement.

### AIM

To evaluate outcomes with interdisciplinary treatment in pediatric patients with pain-related FGIDs and identify patient characteristics that predicted clinical outcomes.

### METHODS

Participants were 392 children, ages 8-18 [M = 13.8; standard deviation (SD) = 2.7], seen between August 1, 2013 and June 15, 2016 in an interdisciplinary APC housed within the Division of Gastroenterology in a medium-sized Midwestern children's hospital. To be eligible, patients had to be 8 years of age or older and have had abdominal pain for  $\geq 8$  wk at the time of initial evaluation. Medical and

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psychosocial data collected as part of standard of care were retrospectively reviewed and analyzed in the context of the observational study. Logistic regression was used to model odds of reporting *vs* never reporting improvement, as well as to differentiate rapid from slower improvers.

## RESULTS

Nearly 70% of patients followed during the study period achieved resolution on at least one of the employed outcome indices. Among those who achieved resolution during follow up, 43% to 49% did so by the first follow up (*i.e.*, within roughly 2 mo after initial evaluation and initiation of interdisciplinary treatment). Patient age, sleep, ease of relaxation, and depression all significantly predicted the likelihood of resolution. More specifically, the odds of clinical resolution were 14% to 16% lower per additional year of patient age ( $P < 0.001$  to  $P = 0.016$ ). The odds of resolution were 28% to 42% lower per 1-standard deviation (SD) increase on a pediatric sleep measure ( $P = 0.006$  to  $P < 0.040$ ). Additionally, odds of clinical resolution were 58% lower per 1-SD increase on parent-reported measure of depression ( $P = 0.006$ ), and doubled in cases where parents agreed that their children found it easy to relax ( $P = 0.045$ ). Furthermore, sleep predicted the rapidity of clinical resolution; that is, the odds of achieving resolution by the first follow up visit were 47% to 60% lower per 1-SD increase on the pediatric sleep measure ( $P = 0.002$ ).

## CONCLUSION

Outcomes for youth with FGIDs may be significantly improved by paying specific attention to sleep, ensuring adequate skills for relaxation, and screening of and referral for treatment of comorbid depression.

**Key words:** Pediatric functional gastrointestinal disorders; Integrated care; Behavioral health consultation; Treatment outcomes; Abdominal pain clinic

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**Core tip:** Naturalistic data collection as part of standard of care in an interdisciplinary specialty clinic allows for early identification of psychosocial factors that complicate the course of pediatric functional gastrointestinal disorders (FGIDs), thereby allowing for proactive intervention. The current study demonstrates that outcomes for youth with FGIDs may be significantly improved by paying specific attention to sleep, ensuring adequate skills for relaxation, and screening of and referral for treatment of comorbid depression.

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## INTRODUCTION

Abdominal pain associated with pediatric functional gastrointestinal disorders (FGIDs) often interferes with daily activities<sup>[1]</sup>, increases risk for psychological comorbidity<sup>[2,3]</sup>, and decreases quality of life<sup>[4]</sup>. It is well accepted that FGIDs are multiply-determined; that is, there is no single and specific cause for the conditions. Instead, a host of biological, psychological and social contributors interact in complicated and varying ways to produce symptoms<sup>[5]</sup>. Historically, treatment for pediatric FGIDs has been conducted in a step-wise fashion, with referral for psychological work up and intervention occurring after the medical evaluation has been “negative” and/or medical treatment has been unsuccessful<sup>[6]</sup>. This approach has the potential to fragment care, and perhaps worse, follow through on referral for psychological services is known to be poor<sup>[7]</sup>.

One alternative to this model is the delivery of co-located medical and psy-



chological care in the context of an interdisciplinary treatment team<sup>[8]</sup>. While the benefits of integrated care have been increasingly well documented in the pediatric health arena<sup>[9]</sup>, interdisciplinary treatment remains the exception, rather than the standard, in the care of pediatric FGID patients. In this study, we sought to add to the small, but growing literature on the efficacy of interdisciplinary treatment for pediatric FGIDs in an outpatient tertiary care setting. We evaluated clinical resolution as measured by change in pain and its associated interference, as well as health-related quality of life. Further, we sought to identify patient characteristics that served as predictors of clinical resolution. We hypothesized that the majority of pediatric patients seen in an interdisciplinary abdominal pain clinic (APC) would demonstrate clinical resolution of symptoms during the study period and that specific psychosocial variables would be significantly predictive of GI symptom improvement.

## MATERIALS AND METHODS

### Participants

Participants were 392 children, ages 8-18 ( $M = 13.8$ ;  $SD = 2.7$ ), seen for initial evaluation (IE) between August 1, 2013 and June 15, 2016 in an interdisciplinary APC (Table 1).

### Study design

Data collected as part of the APC standard of care were retrospectively reviewed and analyzed for the purposes of this investigation, a process approved by the institutional review board. Fifty-three patients (13.7%) were seen for an IE only. This group of patients was generally similar to those who returned for clinic follow up (Table 2). Two statistically significant differences, however, did arise. Patients who attended at least one follow up visit reported at IE that they were less likely to sacrifice important life goals or values in the service of managing pain [Activity Engagement on the Chronic Pain Acceptance Questionnaire-Adolescent, CPAQ-A;  $t = 2.46$  (380),  $P = 0.014$ ] than those who attended the IE only. Additionally, females, compared to males, were disproportionately represented among the patients who attended at least one clinic follow up [ $\chi^2$  (1) = 5.26,  $P < 0.022$ ]. All subsequent reported analyses include only patients who attended at least one follow up visit. The number of follow up visits ranged from 1-15 [median = 3; interquartile range (IQR) 2-6]. The median time between the IE and first follow up was 1.7 mo (IQR 1.4-2.2, range 0.5-22.8); the median time between IE and second follow up was 4.2 mo (IQR 3.2-5.7, range 1.3-33.1).

### Procedure

The APC is an interdisciplinary clinic housed within the Division of Gastroenterology in a medium-sized Midwestern children's hospital in the United States. Patients eligible for care in the APC must be 8 years of age or older and have had abdominal pain for  $\geq 8$  wk. The APC is staffed by two pediatric gastro-enterologists, two advanced practice nurses (APNs), a social worker, two licensed psychologists, one certified biofeedback clinician, and four full-time nurses. A pediatric gastroenterologist and a psychologist jointly conduct the IEs, with both professionals reviewing pre-visit questionnaires and medical history, participating in the development of a treatment plan, and overseeing the in-person clinic visit. Follow up appointments are typically staffed by an APN and a psychologist. For a more in-depth description of the clinic model and typical treatments, see Schurman and Friesen<sup>[10]</sup>.

As part of standard care in the APC, patients and their caregivers complete a battery of assessment measures. An abbreviated battery is collected at all subsequent follow ups, which are scheduled naturalistically as dictated by clinical need. For patients seen during the study period, all clinical information obtained during the IE and subsequent follow up visits was extracted for analyses.

### Measures

**Primary outcomes:** Outcome variables were chosen based on PedIMPACT consensus recommendations<sup>[11]</sup>, and include aspects of the chronic pain itself, as well as physical, emotional, and role functioning. (1) Pediatric Quality of Life Inventory, Version 4.0<sup>[12]</sup> (PedsQL 4.0) is a 23-item measure of health-related quality of life. Physical, Emotional, Social, and School Functioning domain scores and a Total Score can be calculated. Standard scores range from 0 (worst) to 100 (best), and a score of 76 has been established as a critical clinical cutoff<sup>[13]</sup>. We defined self-reported resolution as a PedsQL Self-Report Total  $\geq 76$  and parent-reported resolution as a PedsQL Parent-Report Total score  $\geq 76$ ; (2) Global Improvement Score (GRF) is a categorical, composite rating designed to capture change in pain and functioning since last visit.

**Table 1 Participant characteristics at initial evaluation**

Variable	n	Percent	Range	Mean	SD
Age (yr)	392		8.02-20.52	13.84	2.70
Pain hx (yr)	288		0-17	2.44	3.49
Sex					
Male	103	26.7			
Female	283	73.3			
Ethnicity					
White	342	87.5			
Black or Afr Am	32	8.2			
Amer Indian or Alaskan Native	3	0.8			
Asian Indian	1	0.3			
Chinese	2	0.5			
Filipino	1	0.3			
Japanese	0	0			
Korean	1	0.3			
Vietnamese	1	0.3			
Other Asian	0	0			
Native Hawaiian	0	0			
Guamanian or Chamorro	0	0			
Samoan	0	0			
Other Pacific Islander	1	0.3			
Other	12	3.1			
"Prefer not to answer"	7	1.8			
FD	341	88.3			
IBS	139	36.0			
FAP	14	3.6			
GERD	41	10.6			
Functional constipation	3	0.8			
Functional nausea	0	0			
Abdominal migraine	0	0			
Eosinophilia	301	78.0			

FD: Functional dyspepsia; IBS: Irritable bowel syndrome; FAP: Functional abdominal pain; GERD: Gastroesophageal reflux disease.

Patients are assigned a score of 1 to 5, where 1 = Worse, 2 = Same, 3 = Better (but not meeting criteria for 4 or 5), 4 = Better (symptoms nearly gone or minimal, no interference), and 5 = Better (symptoms gone, no interference). For the purposes of the current study, scores of 4 and 5 were taken to indicate clinical resolution.

**Predictors of outcome:** Predictor variables considered relevant and meaningful<sup>[11]</sup> were selected from among those available in our assessment battery. Additionally, we chose two predictors (*i.e.*, presence of eosinophilia and participation in biofeedback-assisted relaxation training between IE and first follow up) unique to the assessment and intervention strategy in the APC.

(1) Patient age; (2) The Behavior Assessment System for Children, Version 3<sup>[14]</sup> (BASC-3) is a measure of parent- and self-reported adaptive and problem behaviors of youth. T-scores in emotional, behavioral, and social domains are produced and identified as in the normal, at-risk, or clinical range; (3) The Sleep Disturbances Scale for Children<sup>[15]</sup> (SDSC) is a 27-item inventory designed to categorize sleep problems in children. The SDSC produces a Total Score and five subscale scores, disorders of initiating and maintaining sleep, sleep breathing disorders, disorders of arousal, sleep-wake transition disorders, disorders of excessive somnolence, and sleep hyperhidrosis; (4) The Illness Behavior Encouragement Scale<sup>[16]</sup> (IBES) is a 12-item measure that assesses the various ways that parents respond to their children's abdominal pain. Higher scores indicate greater engagement in illness-encouraging behaviors; subscale scores for attention and privileges and release from responsibility<sup>[17]</sup> are calculated; (5) The CPAQ-A<sup>[18]</sup> is a 20-item measure of adolescents'

**Table 2** Baseline comparison of initial evaluation only vs initial evaluation + follow-up patients

Variable	IE only			IE + Follow-Up(s)			P value
	n	Mean	SD	n	Mean	SD	
Age at IE	53	14.33	2.77	333	13.77	2.68	0.157
Pain hx (in yr)	52	3.13	3.97	332	2.62	3.30	0.315
Sex	53 (21 M; 32 F)	-	-	333 (82 M; 251 F)	-	-	0.022 <sup>a</sup>
Eosinophilia	53 (40 Y; 13 N)	-	-	333 (261 Y; 72 N)	-	-	0.635
ActEng T-score	53	51.72	11.53	329	55.75	11.02	0.014 <sup>a</sup>
PainWill T-score	53	56.28	11.31	329	56.31	11.68	0.989
RfR	53	6.32	2.85	332	5.73	2.76	0.148
AttnPriv	53	5.72	4.18	332	5.51	3.46	0.689
% school missed (in previous 4 wk)	42	25.34	27.85	245	22.03	27.44	0.472
Anxiety	53	58.70	12.10	330	58.28	13.73	0.834
Depression	53	56.30	11.92	330	56.72	13.68	0.834
Atypicality	53	52.1	10.29	330	49.72	9.61	0.096
Anxiety_A	53	53.94	11.31	327	54.38	12.27	0.810
Depression_A	53	49.53	9.39	327	50.36	10.87	0.597
Atypicality_A	53	47.58	8.08	327	47.74	9.33	0.907
Social Stress	53	48.08	9.95	327	48.20	10.87	0.935
SDSCTot	53	45.15	9.61	332	45.19	11.74	0.983

<sup>a</sup>Indicates statistical significance. ActEng T-score: Chronic Pain Acceptance Questionnaire-Adolescent Activity Engagement; PainWill T-score: Chronic Pain Acceptance Questionnaire-Adolescent Pain Willingness; RfR: Illness Behavior Encouragement Scale Release from Responsibility; AttnPriv: Illness Behavior Encouragement Scale Attention and Privileges; Anxiety: Behavior Assessment System for Children Parent-Reported Anxiety; Depression: Behavior Assessment System for Children Parent-Reported Depression; Atypicality: Behavior Assessment System for Children Parent-Reported Atypicality; Anxiety\_A: Behavior Assessment System for Children Self-Reported Anxiety; Depression\_A: Behavior Assessment System for Children Self-Reported Depression; Atypicality\_A: Behavior Assessment System for Children Self-Reported Atypicality; Social Stress: Behavior Assessment System for Children Self-Reported Social Stress; SDSCTot: Sleep Disturbances Scale for Children Total Score; IE: Initial evaluation.

acceptance of pain, with higher scores indicating greater pain acceptance. The measure produces two subscales, Pain Willingness (*i.e.*, a recognition that attempts to avoid or control pain are often unproductive) and Activity Engagement (*i.e.*, the pursuit of valued activities regardless of pain); (6) Upset/Relax. Participants and their parents responded with True or False to the following: “I (my child) get (gets) upset too easily” and “It is easy for me (my child) to relax”; (7) School attendance. Patients’ school experience in the 4 wk prior to a follow up visit were coded as: Full-time, on a modified schedule, or not in school; (8) Biofeedback-assisted relaxation training. We recorded endorsement of biofeedback training in the 4 weeks prior to patients’ first follow up visit; (9) Eosinophilia. The presence and location of eosinophilia on endoscopy and colonoscopy were determined by pathology in conjunction with an additional read of biopsies by the physician co-director of the APC (CF).

### Statistical modeling

Using the LOGISTIC Procedure in SAS 9.4, we modeled odds of achieving, at any follow up visit, clinical resolution as defined by GRF score of 4 or 5 as a function of patient age, total years since onset of pain, school status, parent and patient responses to the Upset/Relax questions, SDSC Total score, CPAQ-A subscale scores, IBES subscale scores, various BASC Self-and Parent-Reported subscales, biofeedback prior to first follow up visit, and presence of eosinophilia. We fit two additional logistic regression models to examine odds of achieving the PedsQL cutoff score at a follow up visit as functions of these same predictors. In these models, the analysis was limited to those not already at or above the cutoff score on the PedsQL measure of interest at IE.

In a series of secondary analyses, we examined predictors of rapid resolution among those achieving resolution according to GRF and self- and parent-reported PedsQL criteria. In these, we defined resolution as “rapid” if patients achieved resolution by the 1<sup>st</sup> follow up appointment and “slow” if it occurred by the 2<sup>nd</sup> follow up or anytime thereafter. Given the reduced sample size for these rapid resolution models, we retained only predictors with  $P < 0.10$  from the previous corresponding (Y/N) resolution models in order to limit the number of predictors per case. All analyses were conducted and reviewed by a biomedical statistician (VS).

## RESULTS

### Overall resolution

On the outcome of GRF, 56% of patients reported resolution (*i.e.*, no pain or pain that was nearly gone to minimal with no associated interference) during follow up. Twenty-eight percent reported resolution by the first follow up visit, another 13% by the second follow up visit, and an additional 15% thereafter. Excluding those patients already at or above the clinical cutoff at their IE, 48% of patients, per parents, achieved or exceeded the PedsQL clinical cut off Total score during follow up. Twenty-one percent reported resolution by the first follow up visit, another 15% by the second follow up visit, and an additional 12% thereafter. On the self-report PedsQL, 40% of patients achieved or exceeded the clinical cutoff overall; 17% reported resolution by the first follow up, another 10% by the second follow up, and an additional 13% thereafter.

### Predictors of resolution

Results from the following logistic regression models are summarized in Tables 3-5. Patient age was a significant predictor of resolution on all outcome variables. The odds of resolution according to GRF [odds ratio (OR) = 0.81 (0.73, 0.90),  $P < 0.001$ ] and reaching the parent-reported PedsQL cutoff [OR = 0.85 (0.74, 0.96),  $P = 0.014$ ] and self-reported PedsQL cutoff [OR = 0.86 (0.75, 0.97),  $P = 0.016$ ] were 14% to 19% lower per additional year of age. Sleep also was a significant predictor of resolution. The odds of resolution per the GRF [OR = 0.72 (0.53, 0.98),  $P < 0.040$ ] and reaching the parent-reported PedsQL criterion [OR = 0.58 (0.39, 0.85),  $P = 0.006$ ] were 28% and 42% lower, respectively, per 1-SD increase in the SDSC Total Score. Parent-reported mental health/behavioral concerns were, likewise, predictive of patients achieving resolution. Specifically, the odds of achieving resolution on the self-reported PedsQL variable were 58% lower per 1-SD increase in parent-reported Depression [OR = 0.42 (0.22, 0.76),  $P = 0.006$ ]. Additionally, parents who agreed, "It is easy for my child to relax," had twice the odds [OR = 2.00 (1.02, 3.96),  $P = 0.045$ ] of reporting resolution at follow up according to the parent-reported PedsQL. The odds of achieving resolution according to GRF also were predicted by patients' self-reported Pain Willingness [OR = 0.62 (0.46, 0.82),  $P = 0.001$ ]; surprisingly, the odds of resolution were 38% lower per 1-SD increase in Pain Willingness on the CPAQ-A. Also unexpected, the odds of reaching the parent-reported PedsQL criterion [OR = 2.80 (1.26, 6.43),  $P = 0.013$ ] and self-reported PedsQL criterion [OR = 2.46 (1.09, 5.75),  $P = 0.033$ ] were well over twice as high for those patients whose parents agreed, "My child gets upset too easily".

### Predictors of "Rapid" vs "Slow" resolution

Sleep, again, was determined to be a significant predictor of rapidity of clinical resolution among those achieving resolution. The odds of achieving resolution according to GRF [OR = 0.53 (0.35, 0.78),  $P = 0.002$ ] or reaching the parent-reported QL cutoff [OR = 0.40 (0.22, 0.69),  $P = 0.002$ ] by the first visit were 47% to 60% lower per 1-SD increase in SDSC Total Score.

## DISCUSSION

Nearly 70% of patients followed during the study period achieved resolution on at least one of the employed outcome indices. Among those who achieved resolution during follow up, 43% to 49% did so by the first follow up (*i.e.*, within roughly 2 mo after IE and initiation of interdisciplinary treatment). In general, younger patient age, fewer sleep problems, minimal depression, and reported ease of relaxing at the time of IE significantly predicted patients' clinical resolution. Likewise, better sleep predicted patients' propensity to improve quickly.

Overall, our results are consistent with previous findings. Depression in children with pain-related FGIDs is known to be associated with increased severity of abdominal pain and disability<sup>[19-21]</sup>. Furthermore, evidence suggests that children with both chronic abdominal pain and depression are at risk for continuation of their pain as well as psychiatric disorders in adulthood<sup>[22,23]</sup>. These findings, taken together with our own, provide support to the notion that down mood complicates clinical recovery from GI symptoms and improvement in quality of life in the short-term and, quite likely, in the long-term. Likewise, sleep has been routinely identified as an important factor in pain outcomes for children and adolescents. Specific evidence suggests that: (1) Children and adolescents with pain are likely to experience sleep disturbance; (2) poor sleep in youth with chronic pain is predictive of more pain as well as of impairments in functioning, including quality of life; and (3) intervention with sleep

**Table 3** Logistic regression results for clinical resolution and rapid clinical resolution on global improvement score

Variable	Resolution (area under the curve; AUC = 0.72)		Rapid (vs slow) resolution (AUC = 0.63)	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Age at IE	0.81 (0.73, 0.90)	< 0.001 <sup>a</sup>	0.95 (0.85, 1.06)	0.365
Pain hx (in yr)	1.00 (0.92, 1.08)	0.949		
Eosinophilia	0.84 (0.45, 1.58)	0.593		
ActEng T-score	1.11 (0.83, 1.48)	0.479		
PainWill T-score	0.62 (0.46, 0.82)	0.001 <sup>a</sup>	1.06 (0.74, 1.52)	0.742
RfR	0.85 (0.62, 1.15)	0.288		
AttnPriv	1.02 (0.77, 1.37)	0.865		
School (not in school <i>vs</i> full-time)	1.72 (0.70, 4.45)	0.183		
School (modified schedule <i>vs</i> full-time)	0.81 (0.40, 1.66)	0.243		
Anxiety	1.08 (0.76, 1.54)	0.667		
Depression	1.25 (0.82, 1.93)	0.301		
Atypicality	1.02 (0.72, 1.43)	0.92		
Anxiety_A	1.15 (0.76, 1.75)	0.502		
Depression_A	0.98 (0.64, 1.49)	0.915		
Atypicality_A	1.10 (0.79, 1.56)	0.588		
SDSCTot	0.72 (0.53, 0.98)	0.040 <sup>a</sup>	0.53 (0.35, 0.78)	0.002 <sup>a</sup>
Social Stress	0.66 (0.42, 1.02)	0.064	1.23 (0.86, 1.76)	0.267
Relax_Parent	1.46 (0.81, 2.61)	0.206		
Upset_Parent	0.91 (0.48, 1.75)	0.782		
Relax_Patient	0.99 (0.55, 1.75)	0.961		
Upset_Patient	1.22 (0.66, 2.27)	0.528		
Biofeedback reported at 1st follow up	1.26 (0.76, 2.09)	0.362		

<sup>a</sup>Indicates statistical significance. ActEng T-score: Chronic Pain Acceptance Questionnaire-Adolescent Activity Engagement; PainWill T-score: Chronic Pain Acceptance Questionnaire-Adolescent Pain Willingness; RfR: Illness Behavior Encouragement Scale Release from Responsibility; AttnPriv: Illness Behavior Encouragement Scale Attention and Privileges; Anxiety: Behavior Assessment System for Children Parent-Reported Anxiety; Depression: Behavior Assessment System for Children Parent-Reported Depression; Atypicality: Behavior Assessment System for Children Parent-Reported Atypicality; Anxiety\_A: Behavior Assessment System for Children Self-Reported Anxiety; Depression\_A: Behavior Assessment System for Children Self-Reported Depression; Atypicality\_A: Behavior Assessment System for Children Self-Reported Atypicality; Social Stress: Behavior Assessment System for Children Self-Reported Social Stress; SDSCTot: Sleep Disturbances Scale for Children Total Score; Relax\_Parent: True/False: "It is easy for my child to relax"; Upset\_Parent: True/False: "My child gets upset too easily"; Relax\_Patient: True/False: "It is easy for me to relax"; Upset\_Patient: True/False: "I get upset too easily"; AUC: Area under the curve; CI: Confidence interval.

improves pain outcomes and vice versa<sup>[24]</sup>. Our data uniquely extend the current literature by suggesting that, not only do fewer sleep problems predict clinical resolution and quality of life overall, they predict patients' tendency to report improvement quickly. Recent data also indicate that symptoms of anxiety and depression mediate these pain-sleep relationships<sup>[25]</sup>. Third, pediatric patients with FGIDs often are referred to one of several ancillary services with the most, albeit still limited, empirical support—cognitive-behavioral therapy hypnotherapy, and biofeedback<sup>[26,27]</sup> with the goal of alleviating physical symptoms, *via* general stress management and coping skills training. As such, it is reasonable that children who inherently possess these skills for relaxation and general coping at the outset of treatment for their FGID are more likely to experience clinical resolution of their symptoms.

Contrary to our expectation, higher levels Pain Willingness reported at IE did not predict resolution during the follow up period according to our outcome, GRF. Similar to passive coping strategies, an exclusive focus on elimination of pain as the top priority – that is, an (un) "willingness" to experience pain and regular attempts to avoid or control it – is associated with more depression, anxiety, and functional disability in children and adolescents with chronic pain<sup>[18]</sup>. As such, we anticipated that patients with greater pain willingness at the outset would be more, rather than less, likely to achieve resolution. McCracken *et al.* also reported, however, that while greater acceptance of pain (which includes pain willingness) was associated with less distress and disability, it was not correlated with lower pain intensity. It is possible and even expected, then, that because resolution according to GRF required positive



**Table 4** Logistic regression results for parent-reported pediatric quality of life inventory resolution (score  $\geq 76$ ) and rapid resolution

Variable	Resolution (area under the curve; AUC = 0.78)		Rapid (vs slow) resolution (AUC = 0.69)	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Age at IE	0.85 (0.74, 0.96)	0.014 <sup>a</sup>	0.93 (0.80, 1.09)	0.372
Pain hx (in yr)	0.95 (0.85, 1.05)	0.327		
Eosinophilia	1.32 (0.60, 2.96)	0.492		
ActEng T-score	1.10 (0.78, 1.58)	0.579		
PainWill T-score	1.12 (0.78, 1.59)	0.535		
RfR	0.90 (0.63, 1.3)	0.586		
AttnPriv	0.86 (0.60, 1.25)	0.441		
School (not in school <i>vs</i> full-time)	1.40 (0.50, 4.01)	0.915		
School (modified schedule <i>vs</i> full-time)	1.76 (0.78, 4.04)	0.386		
Anxiety	1.00 (0.64, 1.55)	0.991		
Depression	0.81 (0.49, 1.34)	0.412		
Atypicality	0.92 (0.61, 1.36)	0.662		
Anxiety_A	0.87 (0.52, 1.46)	0.602		
Depression_A	1.18 (0.71, 1.94)	0.516		
Atypicality_A	0.99 (0.67, 1.46)	0.941		
SDSCTot	0.58 (0.39, 0.85)	0.006 <sup>a</sup>	0.40 (0.22, 0.69)	0.002 <sup>a</sup>
Social Stress	0.96 (0.56, 1.64)	0.868		
Relax_Parent	2.00 (1.02, 3.96)	0.045 <sup>a</sup>	0.96 (0.40, 2.27)	0.924
Upset_Parent	2.80 (1.26, 6.43)	0.013 <sup>a</sup>	0.91 (0.38, 2.22)	0.841
Relax_Patient	1.50 (0.74, 3.06)	0.261		
Upset_Patient	0.77 (0.35, 1.69)	0.513		
Biofeedback reported at 1st follow up	1.44 (0.76, 2.77)	0.267		

<sup>a</sup>Indicates statistical significance. ActEng T-score: Chronic Pain Acceptance Questionnaire-Adolescent Activity Engagement; PainWill T-score= Acceptance Questionnaire-Adolescent Pain Willingness; RfR: Illness Behavior Encouragement Scale Release from Responsibility; AttnPriv: Illness Behavior Encouragement Scale Attention and Privileges; Anxiety: Behavior Assessment System for Children Parent-Reported Anxiety; Depression: Behavior Assessment System for Children Parent-Reported Depression; Atypicality: Behavior Assessment System for Children Parent-Reported Atypicality; Anxiety\_A: Behavior Assessment System for Children Self-Reported Anxiety; Depression\_A: Behavior Assessment System for Children Self-Reported Depression; Atypicality\_A: Behavior Assessment System for Children Self-Reported Atypicality; Social Stress: Behavior Assessment System for Children Self-Reported Social Stress; SDSCTot: Sleep Disturbances Scale for Children Total Score; Relax\_Parent: True/False: "It is easy for my child to relax"; Upset\_Parent: True/False: "My child gets upset too easily"; Relax\_Patient: True/False: "It is easy for me to relax"; Upset\_Patient: True/False: "I get upset too easily"; AUC: Area under the curve; CI: Confidence interval.

changes in both pain and disability or functioning, higher pain willingness may not universally predict improvement on this variable.

What the above data suggest is the unequivocal necessity of medical and psychosocial screening, along with combined medical and behavioral intervention, from the outset for pediatric patients with FGIDs. In the APC, we provide broad psychosocial screening as part of the medical history taking and include focused intervention during both IE and follow up visits on sleep hygiene and general stress management. Additionally, we provide targeted behavioral health coaching on topics such as coping, behavioral activation, parenting, medication adherence, and obtaining school support, as well as make recommendations for psychological and psychiatric intervention outside the setting of the APC. This manner of practice is in stark contrast to the typical step-wise intervention (i.e., medical followed by psychological assessment and intervention) that characterizes the bulk of gastroenterology practice at present<sup>[6]</sup>.

The current study possesses a number of strengths. The results presented are the product of naturalistic data collection as part of standard of care in an interdisciplinary specialty clinic. Collecting data in this way allows for early identification of patient factors that can complicate the treatment course of pediatric FGIDs, thereby allowing for proactive intervention. Based on our findings, this is likely to include: intensive targeting of older children and teens to bolster their clinical outcomes (and mitigate the impact of their older age on their tendency to less readily experience clinical resolution), consistent attention paid to sleep quality and quantity during clinic visits, offering of training in specific relaxation training methods, and repeated

**Table 5** Logistic regression results for self-reported pediatric quality of life inventory resolution (score  $\geq 76$ ) and rapid resolution

Variable	Resolution (area under the curve; AUC = 0.80)		Rapid (vs slow) resolution (AUC = 0.65)	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Age at IE	0.86 (0.75, 0.97)	0.016 <sup>a</sup>	1.01 (0.87, 1.18)	0.888
Pain hx (in yr)	1.03 (0.92, 1.14)	0.610		
Eosinophilia	0.89 (0.37, 2.08)	0.786		
ActEng T-score	1.11 (0.76, 1.62)	0.574		
PainWill T-score	1.01 (0.70, 1.45)	0.975		
RfR	0.88 (0.60, 1.28)	0.514		
AttnPriv	0.75 (0.51, 1.10)	0.143		
School (not in school <i>vs</i> full-time)	0.83 (0.28, 2.42)	0.663		
School (modified schedule <i>vs</i> full-time)	1.12 (0.46, 2.77)	0.672		
Anxiety	1.04 (0.65, 1.66)	0.872		
Depression	0.42 (0.22, 0.76)	0.006 <sup>a</sup>	0.71 (0.32, 1.49)	0.378
Atypicality	1.42 (0.92, 2.20)	0.113		
Anxiety_A	1.15 (0.68, 1.97)	0.597		
Depression_A	0.67 (0.36, 1.22)	0.206		
Atypicality_A	0.73 (0.48, 1.07)	0.115		
SDSCTot	0.78 (0.51, 1.18)	0.246		
Social Stress	0.68 (0.38, 1.20)	0.190		
Relax_Parent	1.50 (0.74, 3.06)	0.260		
Upset_Parent	2.46 (1.09, 5.75)	0.033 <sup>a</sup>	1.31 (0.44, 4.07)	0.632
Relax_Patient	1.21 (0.60, 2.46)	0.590		
Upset_Patient	1.94 (0.93, 4.11)	0.080	0.41 (0.15, 1.09)	0.076
Biofeedback reported at 1st follow up	0.82 (0.42, 1.57)	0.543		

<sup>a</sup>Indicates statistical significance. ActEng T-score: Chronic Pain Acceptance Questionnaire-Adolescent Activity Engagement; PainWill T-score= Acceptance Questionnaire-Adolescent Pain Willingness; RfR: Illness Behavior Encouragement Scale Release from Responsibility; AttnPriv: Illness Behavior Encouragement Scale Attention and Privileges; Anxiety: Behavior Assessment System for Children Parent-Reported Anxiety; Depression: Behavior Assessment System for Children Parent-Reported Depression; Atypicality: Behavior Assessment System for Children Parent-Reported Atypicality; Anxiety\_A: Behavior Assessment System for Children Self-Reported Anxiety; Depression\_A: Behavior Assessment System for Children Self-Reported Depression; Atypicality\_A: Behavior Assessment System for Children Self-Reported Atypicality; Social Stress: Behavior Assessment System for Children Self-Reported Social Stress; SDSCTot: Sleep Disturbances Scale for Children Total Score; Relax\_Parent: True/False: "It is easy for my child to relax"; Upset\_Parent: True/False: "My child gets upset too easily"; Relax\_Patient: True/False: "It is easy for me to relax"; Upset\_Patient: True/False: "I get upset too easily"; AUC: Area under the curve; CI: Confidence interval.

screening and referral for pediatric and adolescent depression. Second, data collection at each and every visit allows provider teams to be clinically nimble and adapt to changes in patients' presentations more quickly than would be possible without this information. Finally, repeated data collection at naturalistic time points during provides ample statistical power for modeling complex clinical questions whose answers reflect the real-world waxing and waning of symptoms and associated circumstances, thereby decreasing the chance of missing naturally occurring symptomatic variability.

These strengths notwithstanding, the study possesses limitations worth mention. To start, the retrospective, uncontrolled nature of the study design does not allow casual inferences to be made about the specific impact of our interdisciplinary, standard of care intervention. Second, given the number of predictors included (and, thus, hypotheses reported) in our analyses, statistical significance should be interpreted with caution. We report p-values not as arbiters of clinical importance, but as aids in identifying effects that are unlikely to be attributable solely to chance. Finally, and perhaps most important, because data were collected naturalistically and not at predetermined time points, the interpretation of "missing" data becomes complicated. In the event that patients do not attend scheduled follow up visits because they are well, "missing" data may, in fact, signal improvement that is unreported or undetected. We also employed a strict definition of resolution on the GRF, requiring that symptoms, even if improved, were causing no impairment in patients' functioning. Thus, even if patients identified themselves as better, but continued to experience even mild impairment in their functioning due to abdominal

pain (*i.e.*, GRF = 3), our analyses classified these as instances of non-resolution. As such, we argue that our results are likely to be rather conservative estimates of our patients' improvement, though additional data would be needed to confirm this claim.

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## ARTICLE HIGHLIGHTS

### Research background

Abdominal pain characteristic of pediatric functional gastrointestinal disorders (FGIDs) is known to be associated with a high degree of psychosocial comorbidity and to persist into adulthood without intervention. Likewise, it is well accepted that a host of biological, psychological, and social factors contribute and interact in complicated and varying ways to produce the various FGID phenotypes. Historically, treatment for pediatric FGIDs has been conducted such that, following a "negative" medical evaluation and/or unsuccessful medical treatment, referrals to mental health providers are made and relevant treatments undertaken. One alternative to this model is the delivery of co-located medical and psychological care in the context of an interdisciplinary treatment team. Although the benefits of integrated care are well documented in pediatrics, interdisciplinary care remains the exception, rather than the standard, in the care of pediatric FGID patients. The current study aims to address this current gap in the existing literature.

### Research motivation

In an effort to measure and improve upon clinical change in both medical and psychosocial outcomes in pediatrics FGIDs, we employed naturalistic data collection as part of standard of care in an interdisciplinary specialty clinic. In so doing, we collected a rich and diverse data set that allowed us to evaluate patients' clinical resolution, as well as identify factors that complicate symptom improvement. This is significant in that it adds to the small, existing literature on the efficacy of interdisciplinary treatment for pediatric FGIDs in an outpatient tertiary care setting. Furthermore, identification of psychosocial factors that delay or prevent symptom improvement sets the stage for early, proactive intervention.

### Research objectives

The primary research objectives included: evaluation of outcomes with interdisciplinary treatment in pediatric patients with pain-related FGIDs, and identification of patient characteristics that predicted clinical outcomes.

### Research methods

Study participants were 392 children, ages 8-18 ( $M = 13.8$ ;  $SD = 2.7$ ), seen between August 1, 2013 and June 15, 2016 in an interdisciplinary APC housed within the Gastroenterology Division of a medium-sized children's hospital in the United States. To be eligible for the study, patients had to be 8 years of age or older and have had abdominal pain for  $\geq 8$  wk at the time of initial evaluation. Medical and psychosocial data collected naturalistically as part of standard of care were retrospectively reviewed and analyzed. Logistic regression was used to model odds of reporting vs. never reporting improvement, as well as to differentiate rapid from slower improvers. Collecting data in this way allows for early identification of patient factors that can complicate the treatment course of pediatric FGIDs, thereby allowing for proactive intervention. Second, data collection at each and every visit allows provider teams to be clinically nimble and adapt to changes in patients' presentations more quickly than would be possible without this information. Finally, repeated data collection at naturalistic time points during provides ample statistical power for modeling complex clinical questions whose answers reflect the real-world waxing and waning of symptoms and associated circumstances, thereby decreasing the chance of missing naturally occurring symptomatic variability.

### Research results

Nearly 70% of patients followed during the study period achieved clinical resolution on at least one of the employed outcome indices. Among those who achieved resolution during follow up, close to half did so within roughly 2 mo after initial evaluation and initiation of interdisciplinary treatment. Patient age, sleep, ease of relaxation, and depression all significantly predicted the likelihood of resolution, with older age, poor sleep, difficulty relaxing, and the presence of depression predicting worse outcomes. Poor sleep also was found to significantly predict the rapidity of clinical resolution such that it delayed clinical resolution of symptoms beyond the first follow up visit. The identification of the relationships between patient age, sleep, ease of relaxation, and depression and FGID symptom improvement is a critical first step in crafting the most effective biopsychosocial interventions for this complex set of diagnoses.

### Research conclusions

As anticipated, a great majority of patients treated in the context of an interdisciplinary model of care for chronic abdominal pain demonstrated improvement. In addition, unique psychosocial characteristics were able to be identified that uniquely predicted the presence and pace of positive outcomes. Based on our findings, clinical outcomes among youth with pediatric FGIDs are likely facilitated by intensive targeting of older children and teens to bolster their clinical outcomes, consistent attention paid to sleep quality and quantity during clinic visits, offering of training in specific relaxation training methods, and repeated screening and referral for pediatric and adolescent depression. Furthermore, these findings highlight the need for continued inquiry into the benefit and necessity of concurrent medical and psychosocial screening and intervention as standard of care for all for children affected by FGIDs.

### Research perspectives

Use of naturalistically collected data in the context of an observational study provides rich and unique clinical and research opportunities. Data collected as standard of care in a busy clinic provides opportunities for individualized, in-the-moment intervention with patients as they present, as well as the ability of researchers to identify patterns among groups of patients. In the case of the current study, we were able to identify behavioral factors that, if addressed, have the potential to increase the likelihood of clinical symptom resolution among youth with FGIDs. Future investigations would benefit from the use of controlled research designs wherein researchers compared standard medical care to interdisciplinary care.

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## Recent advances in endoscopic retrograde cholangiopancreatography in Billroth II gastrectomy patients: A systematic review

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### Abstract

#### BACKGROUND

Endoscopic retrograde cholangiopancreatography (ERCP) in patients with Billroth II gastrectomy has been considered a challenging procedure due to the surgically altered gastrointestinal anatomy. However, there has been a paucity of comparative studies regarding ERCP in Billroth II gastrectomy cases because of procedure-related morbidity and mortality and practical and ethical limitations. This systematic and comprehensive review was performed to obtain a recent perspective on ERCP in Billroth II gastrectomy patients.

#### AIM

To systematically review the literature regarding ERCP in Billroth II gastrectomy patients.

#### METHODS

A systematic review was performed on the literature published between May 1975 and January 2019. The following electronic databases were searched: PubMed, EMBASE, and Cochrane Library. The outcomes of successful afferent loop intubation and successful selective cannulation and occurrence of adverse events were assessed.

#### RESULTS

A total of 43 studies involving 2669 patients were included. The study designs were 36 (83.7%) retrospective cohort studies, 4 (9.3%) retrospective comparative studies, 2 (4.7%) prospective comparative studies, and 1 (2.3%) prospective cohort study. Of a total of 2669 patients, there were 1432 cases (55.6%) of side-viewing endoscopy, 664 (25.8%) cases of forward-viewing endoscopy, 171 (6.6%)

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cases of balloon-assisted enteroscopy, 169 (6.6%) cases of anterior oblique-viewing endoscopy, 64 (2.5%) cases of dual-lumen endoscopy, 31 (1.2%) cases of colonoscopy, and 14 (0.5%) cases of multiple bending endoscopy. The overall success rate of afferent loop intubation was 91.3% (2437/2669), and the overall success rate of selective cannulation was 87.9% (2346/2437). A total of 195 cases (7.3%) of adverse events occurred. The success rates of afferent loop intubation and the selective cannulation rate for each type of endoscopy were as follows: side-viewing endoscopy 98.2% and 95.3%; forward-viewing endoscopy 97.4% and 95.2%; balloon-assisted enteroscopy 95.4% and 97.5%; oblique-viewing endoscopy 94.1% and 97.5%; and dual-lumen endoscopy 82.8% and 100%, respectively. The rate of bowel perforation was slightly higher in side-viewing endoscopy (3.6%) and balloon-assisted enteroscopy (4.1%) compared with forward-viewing endoscopy (1.7%) and anterior oblique-viewing endoscopy (1.2%). Mortality only occurred in side-viewing endoscopy ( $n = 9$ , 0.6%).

## CONCLUSION

The performance of ERCP in the Billroth II gastrectomy population has been improving with choice of various type of endoscope and sphincter management. More comparative studies are needed to determine the optimal strategy to perform safe and effective ERCP in Billroth II gastrectomy patients.

**Key words:** Endoscopic retrograde; Cholangiopancreatography; Therapeutic; Endoscopy; Billroth II operation; Adverse event; Systematic review

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**Core tip:** Endoscopic retrograde cholangiopancreatography (ERCP) in Billroth II gastrectomy anatomy has been considered a difficult procedure due to the surgical alteration. To date, there has been a paucity of comparative studies regarding ERCP in Billroth II gastrectomy patients. In current study, we systematically and comprehensively reviewed the literatures regarding ERCP in Billroth II gastrectomy cases. The performance of ERCP in the Billroth II gastrectomy has been improving with choice of various type of endoscope and sphincter therapy. More comparative studies are required to perform effective and safe ERCP in Billroth II gastrectomy population.

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## INTRODUCTION

Billroth II gastrectomy commonly encounters a challenging surgically altered anatomy when performing endoscopic retrograde cholangiopancreatography (ERCP). The difficulties in performing ERCP in patients with Billroth II gastrectomy include the identification and intubation of the afferent loop, visualization of the papilla, selective cannulation of the desired biliary or pancreatic duct, and sphincter therapy due to the reverse direction of the papilla<sup>[1,2]</sup>. As a result, the safety and failure of ERCP have always been a major concern in Billroth II gastrectomy patients.

For successful and safe ERCP in Billroth II gastrectomy patients, there have been many choices for the selection of endoscopy other than conventional side-viewing endoscopy, such as forward-viewing endoscopy (with or without cap-fitting)<sup>[3,4]</sup>, balloon-assisted enteroscopy (single-balloon or double-balloon)<sup>[5,6]</sup>, anterior oblique-viewing endoscopy (with or without overtube-assisted)<sup>[7,8]</sup>, dual-lumen endoscopy<sup>[9]</sup>, and multiple bending endoscopy<sup>[10]</sup>; choices for sphincter therapy, such as endoscopic sphincterotomy (EST), endoscopic papillary balloon dilation (EPBD), and endoscopic papillary large balloon dilation (EPLBD)<sup>[11-13]</sup>; and choices for accessories, such as a needle knife (NK) and rotatable or dedicated inverted papillotome<sup>[14,15]</sup>.

To date, there has been a paucity of comparative studies regarding ERCP in Billroth

II gastrectomy cases because of procedure-related morbidity and mortality and practical and ethical limitations. To obtain a more recent perspective on ERCP in Billroth II gastrectomy, we systematically and comprehensively reviewed the literature regarding ERCP in Billroth II gastrectomy patients. In detail, the aims of our study were: (1) To assess the success rate of afferent loop intubation, the success rate of selective cannulation, and rate of adverse events in ERCP in Billroth II gastrectomy patients; (2) To assess these outcomes according to each type of endoscopy; (3) To assess clinical efficacy according to each type of sphincter management methods.

## MATERIALS AND METHODS

### Search strategy and study selection

This systematic review was conducted according to the PRISMA guidelines<sup>[16]</sup>. Electronic databases, including MEDLINE (PubMed), EMBASE, and Cochrane Library, were searched for all studies published from May 1975 to January 2019. The search terms included “Billroth II gastrectomy” or “Billroth II operation,” or “gastrectomy” and “endoscopic retrograde cholangiopancreatography” or “endoscopic retrograde” combined with “cholangiopancreatography,” or “ERCP.” Two investigators (T.Y.P. and T.J.S.) independently performed the search of the electronic databases and assessed the eligibility of all the studies searched from the databases according to the predetermined selection criteria. Disagreements between evaluators were resolved through discussion.

Studies were included in the systematic review if they met all of the following criteria: (1) Relevant clinical studies of ERCP in patients with prior Billroth II gastrectomy; (2) Studies that enrolled at least 10 Billroth II gastrectomy patients; (3) Studies with full text available; (4) Studies with available information on the patient number, indications for the ERCP, type of endoscopy, type of sphincter therapy, success rate of afferent loop intubation, success rate of selective cannulation, occurrence of adverse events including bowel perforation, post-ERCP pancreatitis, bleeding, cardiopulmonary events and mortality; and (5) Studies written in English. Studies were excluded from the current review if they met any of the following criteria: (1) Irrelevancy to ERCP in Billroth II gastrectomy patients; (2) Fewer than ten patients; (3) Review, abstract only article, commentary, and letter; (4) Non-human study; and (5) Languages other than English.

Data of the sample size, study design, indications for the procedure, type of endoscopy (side-viewing endoscopy, forward-viewing endoscopy, double-balloon enteroscopy, single-balloon enteroscopy, anterior oblique-viewing endoscopy, overtube-assisted endoscopy, multi-bending endoscopy, or dual-lumen endoscopy), type of sphincter therapy (EST, EPBD, EPLBD, NK), success rate of afferent loop intubation, success rate of selective cannulation, occurrence of adverse events including bowel perforation, post-ERCP pancreatitis, procedure-related bleeding, cardiopulmonary events, and mortality were extracted from the included studies. Subgroup analysis of successful afferent loop intubation, successful selective cannulation, bowel perforation, post-ERCP pancreatitis, bleeding, and mortality according to the type of endoscopy was performed. And clinical outcomes according to the type of sphincter therapy was also evaluated. Clinical success was defined as the achievement of the planned therapeutic goals including bile duct stone clearance, endobiliary biopsy, biliary stent or nasobiliary catheter insertion. Data extraction was carried out by two independent reviewers (T.Y.P. and T.J.S.) using a standardized table. Discrepancies were resolved by discussion.

### Statistical analysis

The primary outcome was to assess the efficacy of ERCP in Billroth II gastrectomy patients by afferent loop intubation and the selective cannulation of the desired duct as well as the safety according to procedure-related adverse events, such as bowel perforation, post-ERCP pancreatitis, bleeding, cardiopulmonary events, and mortality. The secondary outcome was to compare the rate of afferent loop intubation, selective cannulation, and adverse events according each type of endoscope. The categorical variables were reported as the frequency with respective proportions (percentages). The pooled rate of outcome measures was calculated by dividing the percentage of patients or procedures from the included studies.

## RESULTS

### Literature search and identification of relevant studies

The flow diagram of the study identification, screening, eligibility, and inclusion process is shown in [Figure 1](#). A total of 344 studies were identified through an electronic search of 3 databases and manual search of the relevant bibliographies. Of them, 79 duplicate studies were removed during the initial screening. Then, through a review of the titles and abstracts, 100 studies irrelevant to ERCP in Billroth II gastrectomy patients were excluded. After a thorough review of 265 relevant studies, 222 studies were excluded from the systematic review. The reasons for study exclusion were as follows: Case report ( $n = 28$ ), languages other than English ( $n = 26$ ), fewer than 10 patients ( $n = 25$ ), review ( $n = 15$ ), abstract ( $n = 13$ ), letter ( $n = 7$ ), commentary ( $n = 6$ ), and non-human study ( $n = 2$ ). The remaining 43 studies were included in the final analysis.

### **Characteristics of the studies included in the final review**

The characteristics of the 43 studies are listed in [Table 1](#). The published year ranged from 1984 to 2018. Most of the studies were retrospective single-arm studies, and the most common indications for ERCP were common bile duct (CBD) stones and pancreaticobiliary malignancies. There were six studies that included more than 100 Billroth II gastrectomy patients and, among them, three studies<sup>[17-19]</sup> were published in recent years. The detailed characteristics of the recently published studies including more than 100 Billroth II gastrectomy cases are summarized in [Table 2](#).

### **Results of the systematic review**

The results of the current systematic review are shown in [Table 3](#). Of the 43 included studies, there were 36 (83.7%) retrospective cohort studies, 4 (9.3%) retrospective comparative studies, 2 (4.7%) prospective comparative studies, and 1 (2.3%) prospective cohort study. There were 2669 identified patients in total. Conventional side-viewing endoscopy ( $n = 1432$ , 55.6%) and forward-viewing endoscopy with or without cap-fitting ( $n = 664$ , 25.8%) were the most frequently used types of endoscopy when performing ERCP in Billroth II gastrectomy patients. The other types of endoscopy that were used were balloon-assisted enteroscopy in 171 cases (6.6%), anterior oblique-viewing endoscopy in 169 cases (6.6%), dual-lumen endoscopy in 64 cases (2.5%), colonoscopy in 31 (1.2%), and multiple bending endoscopy in 14 cases (0.5%). The overall success rate of afferent loop intubation was 91.3% (2437/2669), and the overall success rate of selective cannulation was 87.9% (2346/2437). A total of 195 cases (7.3%) of adverse events occurred. These events were bowel perforations in 74 cases (2.8%), post-ERCP pancreatitis in 65 cases (2.4%), bleeding in 37 cases (1.4%), mortality in 9 cases (0.3%), cholangitis in 7 cases (0.1%), respiratory insufficiency in 1 case (0.04%), aspiration pneumonia in 1 case (0.04%), and cholecystitis in 1 case (0.04%). All the mortality cases ( $n = 9$ , 0.3%) occurred in procedures using conventional side-viewing endoscopy.

### **Subgroup analysis**

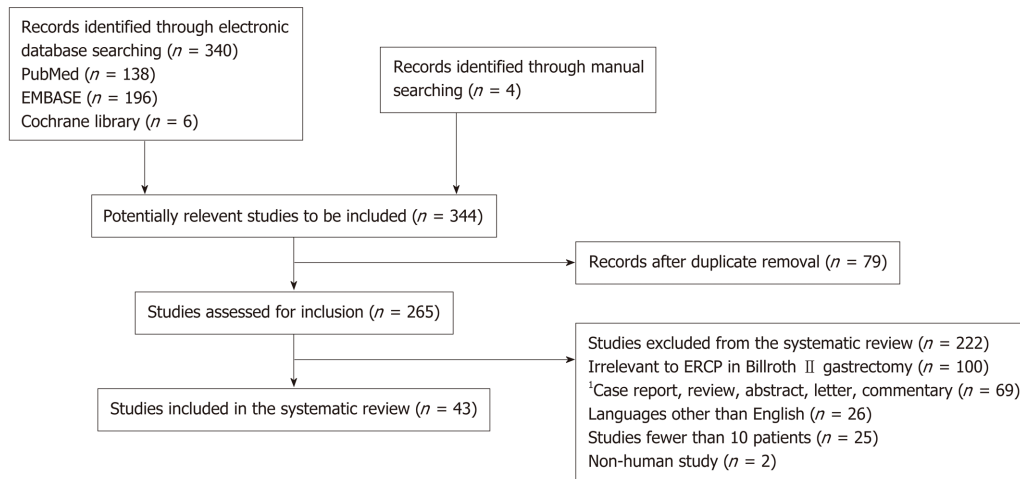
The subgroup analysis according to the type of endoscopy is summarized in [Table 4](#). The success rates of afferent loop intubation by each type of endoscopy ranged from 82.8% to 98.2%. The success rates of selective cannulation ranged from 95.2% to 100%. The occurrence rate of adverse events by each type of endoscopy ranged from 3.6% to 7.9%. The rates of afferent loop intubation, selective cannulation, and adverse events were similar between side-viewing endoscopy and forward-viewing endoscopy, which are the most frequently used types of endoscopy in ERCP in patients with Billroth II gastrectomy. The rates of bowel perforation were slightly higher in side-viewing endoscopy (3.6%) and balloon-assisted enteroscopy (4.1%) compared with forward-viewing endoscopy (1.7%) and anterior oblique-viewing endoscopy (1.2%). Mortality only occurred in side-viewing endoscopy ( $n = 9$ , 0.6%).

The subgroup analysis by each type of sphincter management summarized in [Table 5](#). The clinical success rates of achievement for the planned therapeutic goals according to the sphincter management ranged from 85.8% to 93.6%. The overall rate of adverse events according to the sphincter therapy ranged from 5.8% to 8.5%. The rate of bowel perforation ranged from 1.3% to 3.5%. The most cases of post-ERCP pancreatitis occurred in patients who underwent EPBD (6.5%). Most of the bleeding occurred in whom EST was used (EST,  $n = 25$ ; EST+EPBD,  $n = 8$ ; EPBD,  $n = 3$ ).

## **DISCUSSION**

The gastric bypass surgery was first introduced in 1879 by Jules Emile Pean and 1880 by Ludwik Rydygier<sup>[20]</sup>. The gastrectomy with gastrojejunal anastomosis (Billroth II gastrectomy) is the most modern form of gastric bypass surgery, which was first performed in 1885 by Theodor Billroth<sup>[21]</sup>. Now, Billroth II gastrectomy has been





**Figure 1** Flow diagram of the study.<sup>1</sup>Case report ( $n = 28$ ), review ( $n = 15$ ), abstract ( $n = 13$ ), letter ( $n = 7$ ), and commentary ( $n = 6$ ).

widely used to treat gastric malignancy, refractory peptic ulcer disease with pyloric stenosis, or peptic ulcer perforation<sup>[22]</sup>. There are several types of Billroth II anastomosis according to reconstruction following partial gastrectomy such as antecolic or retrocolic, anisoperistaltic or isoperistaltic with or without Roux-en-Y anastomosis.

ERCP in Billroth II gastrectomy patients is a challenging procedure. The difficulties in performing ERCP in Billroth II gastrectomy are selective intubation of the endoscope into the afferent loop due to the acute angulation of the remnant stomach and small bowel, identification of papilla behind the mucosal fold, selective cannulation of the desired pancreaticobiliary duct, and optimal sphincter therapy due to the inverted position<sup>[23]</sup>. Particularly, because of procedure-related morbidity and mortality, there has been a paucity of prospective controlled studies in this population, and the treatment strategy or instrument decision, including the choice of endoscope or sphincter therapy, has been determined according to the endoscopists' preference based on their education and experience.

The choice of endoscopy has always been a matter of controversy, and there is no consensus on the issues. Experienced endoscopists usually recommend using a conventional side-viewing endoscope because it has an elevator and a large working channel. However, a side-viewing endoscope has some limitations when used on Billroth II gastrectomy patients because of its limited visibility due to presenting a side view, rigidity and relatively large diameter of the scope. Therefore, in Billroth II gastrectomy patients, ERCP using a side-viewing endoscope may sometimes be difficult, and it may be associated with a risk of small bowel or an anastomosis site perforation, particularly for inexperienced endoscopists. By contrast, insertion of a forward-viewing endoscope may be relatively easy and safe, and various interventions can also be performed safely. Consequently, the use of a forward-viewing endoscope with or without cap-fitting has become more frequent and now is the second most common type of endoscope for ERCP in Billroth II gastrectomy patients despite its disadvantages, such as absence of an elevator, small working channel, and difficulty in obtaining an en face view of the papilla (Figures 2 and 3).

Recently, a meta-analysis<sup>[24]</sup> that focused on the efficacy and safety of forward-viewing endoscopy for ERCP in Billroth II gastrectomy compared with conventional side-viewing endoscopy was reported. The meta-analysis showed a higher success rate for afferent loop intubation in forward-viewing endoscopy (with or without cap-fitting) compared to that for conventional side-viewing endoscopy (90.3% *vs* 86.8%). Furthermore, the success rate of selective cannulation in cap-fitting forward-viewing endoscopy has been reported up to 93.7%. This result suggested that forward-viewing endoscopy with or without cap-fitting can be a potential alternative type of endoscopy for ERCP in Billroth II gastrectomy cases, particularly when conventional side-viewing endoscopy fails and balloon-assisted enteroscopy is unavailable. A forward-viewing endoscopy with or without cap-fitting could be the initial choice of endoscopy for an inexperienced endoscopist to minimize the risk of adverse events, such as bowel perforation. However, the studies included in this meta-analysis were non-comparative and had a retrospective design, and therefore, the applicability of their pooled estimate results to general practice might be limited.



Table 1 Characteristics of the 43 studies included in the systematic review

Study	Study design	No.	Indications for ERCP	Type of endoscope	Sphincter therapy	A-loop intubation, No. (%)	Selective cannulation, No. (%)	Adverse events
Forbes and Cotton <sup>[1]</sup> , 1984	Retrospective cohort	53	N/A	S	EST	45/53 (84.9)	35/45 (77.8)	Bowel perforation ( <i>n</i> = 1, 1.9%)
Osnes <i>et al</i> <sup>[2]</sup> , 1986	Retrospective cohort	147	N/A	S	EST	134/147 (91.2)	134/134 (100)	Bowel perforation ( <i>n</i> = 1, 0.7%) Pancreatitis ( <i>n</i> = 1, 0.7%) Bleeding ( <i>n</i> = 1, 0.7%) Mortality ( <i>n</i> = 2, 1.4%)
Hintze <i>et al</i> <sup>[31]</sup> , 1997	Retrospective cohort	59	CBD stone  Papillary stenosis Tumor stenosis Juxtapapillary diverticulum	S	EST	54/59 (91.5)	54/54 (100)	Bowel perforation ( <i>n</i> = 1, 1.7%) Mortality ( <i>n</i> = 1, 1.7%)
Kim <i>et al</i> <sup>[3]</sup> , 1997	Prospective comparative	45	N/A	F ( <i>n</i> = 23)  S ( <i>n</i> = 22)	EST±NK	44/45 (97.8)	36/44 (80.0)	Bowel perforation ( <i>n</i> = 4, 8.9%) Pancreatitis ( <i>n</i> = 3, 2.2%)
Lin <i>et al</i> <sup>[32]</sup> , 1999	Retrospective cohort	56	CBD stone CBD dilation RUQ pain with cholestasis	F	EST	43/56 (76.6)	35/43 (81.3)	Bleeding ( <i>n</i> = 3, 5.4%)
Faylona <i>et al</i> <sup>[33]</sup> , 1999	Retrospective cohort	110	Cholangitis ( <i>n</i> = 58)  CBD stone ( <i>n</i> = 41) Jaundice ( <i>n</i> = 28) CBD dilation ( <i>n</i> = 19) Pancreatitis ( <i>n</i> = 9) Others ( <i>n</i> = 30)	S	EST	<sup>2</sup> 132/185 (71.4)	<sup>2</sup> 122/132 (92.4)	Bowel perforation ( <i>n</i> = 11, 5.9%) Pancreatitis ( <i>n</i> = 1, 0.5%)  Bleeding ( <i>n</i> = 3, 1.6%) Mortality ( <i>n</i> = 2, 1.1%)
Bergman <i>et al</i> <sup>[11]</sup> , 2001	Prospective comparative	34	CBD stone ( <i>n</i> = 34)	S	EST/EPBD	N/A	28/34 (82.4)	Bowel perforation ( <i>n</i> = 1, 2.9%) Pancreatitis ( <i>n</i> = 1, 2.9%) Bleeding ( <i>n</i> = 3, 8.8%) Respiratory insufficiency ( <i>n</i> = 1, 2.9%)
Swarnkar <i>et al</i> <sup>[34]</sup> , 2005	Retrospective cohort	41	CBD stone ( <i>n</i> = 16) CBD dilation ( <i>n</i> = 9) Pancreatitis ( <i>n</i> = 4) Gastric cancer ( <i>n</i> = 3)	S	EST	<sup>2</sup> 42/48 (87.5)	<sup>2</sup> 41/42 (97.6)	Bowel perforation ( <i>n</i> = 1, 2.1%)

			Pancreatic cancer ( <i>n</i> = 2)					
			Others ( <i>n</i> = 7)					Bleeding ( <i>n</i> = 2, 4.2%)
Kikuyama <i>et al</i> <sup>[35]</sup> , 2005	Retrospective cohort	24	CBD stone ( <i>n</i> = 14)	AOE	EST	24/24 (100)	22/24 (91.7)	Bowel perforation ( <i>n</i> = 1, 4.2%)
			Pancreaticobiliary malignancy ( <i>n</i> = 8)					Pancreatitis ( <i>n</i> = 1, 4.2%)
			Others ( <i>n</i> = 2)					Bleeding ( <i>n</i> = 1, 4.2%)
Çiçek <i>et al</i> <sup>[36]</sup> , 2006	Retrospective cohort	52	CBD stone ( <i>n</i> = 27)	S	EST±NK	45/52 (94.2)	43/45 (95.6)	Bowel perforation ( <i>n</i> = 6, 11.5%)
			Jaundice ( <i>n</i> = 11)					Bleeding ( <i>n</i> = 3, 1.6%)
			Pancreaticobiliary malignancy ( <i>n</i> = 10)					( <i>n</i> = 2, 3.8%)
			Bile leakage ( <i>n</i> = 2)					
			Others ( <i>n</i> = 2)					
Park <i>et al</i> <sup>[37]</sup> , 2007	Retrospective cohort	10	CBD stone ( <i>n</i> = 9)	<sup>1</sup> F	EST	10/10 (100)	10/10 (100)	None
			CBD stricture ( <i>n</i> = 1)					
Dolay and Soyulu <sup>[38]</sup> , 2008	Retrospective cohort	11		S	EST	11/11 (100)	11/11 (100)	None
Nakahara <i>et al</i> <sup>[39]</sup> , 2009	Retrospective comparative	43	CBD stone ( <i>n</i> = 43)	AOE	EST/EPBD±NK	38/43 (88.4)	36/38 (94.7)	None
Koo <i>et al</i> <sup>[10]</sup> , 2009	Retrospective cohort	14	CBD stone ( <i>n</i> = 8)	Multiple bending endoscope	EST/EPBD	14/14 (100)	13/14 (92.9)	None
			Biliary pancreatitis ( <i>n</i> = 2)					
			Pancreaticobiliary malignancy ( <i>n</i> = 3)					
			Bile leakage after cholecystectomy ( <i>n</i> = 1)					
Shimatani <i>et al</i> <sup>[40]</sup> , 2009	Retrospective cohort	17	N/A	DBE	EST/EPBD	<sup>2</sup> 22/22 (100)	<sup>2</sup> 22/22 (100)	None
Kikuyama <i>et al</i> <sup>[41]</sup> , 2009	Retrospective cohort	11	CBD stone ( <i>n</i> = 8)	AOE with over tube	EST/EPBD	<sup>2</sup> 10/15 (66.7)	<sup>2</sup> 10/10 (100)	None
			Pancreaticobiliary malignancy ( <i>n</i> = 3)					
			Chronic pancreatitis ( <i>n</i> = 1)					
Lin <i>et al</i> <sup>[42]</sup> , 2010	Retrospective cohort	32	N/A	S ( <i>n</i> = 22) DBE ( <i>n</i> = 8)	EPBD	30/32 (68.8)	28/30 (93.3)	Bowel perforation ( <i>n</i> = 2, 6.3%)
Itoi <i>et al</i> <sup>[12]</sup> , 2010	Retrospective cohort	11	CBD stone ( <i>n</i> = 11)	F ( <i>n</i> = 8) S ( <i>n</i> = 1) AOE ( <i>n</i> = 1) SBE ( <i>n</i> = 1)	EST+EPLBD	11/11 (100)	11/11 (100)	None
Lee <i>et al</i> <sup>[30]</sup> , 2012	Retrospective cohort	13	CBD stone ( <i>n</i> = 13)	<sup>1</sup> F	EPLBD	13/13 (100)	12/13 (92.3)	Bleeding ( <i>n</i> = 1, 7.7%)
Byun <i>et al</i> <sup>[43]</sup> , 2012	Retrospective cohort	46	CBD stone ( <i>n</i> = 37)	F	EST+EPBD	42/46 (91.3)	42/42 (100)	Bowel perforation ( <i>n</i> = 1, 2.2%)

			Pancreatico-biliary malignancy ( <i>n</i> = 5)					Pancreatitis ( <i>n</i> = 1, 2.2%)
			Benign biliary stricture ( <i>n</i> = 4)					
Choi <i>et al</i> <sup>[44]</sup> , 2012	Retrospective comparative	26	CBD stone ( <i>n</i> = 26)	S ( <i>n</i> = 13) F ( <i>n</i> = 13)	EST±EPBD	26/26 (100)	26/26 (100)	None
Kianicka <i>et al</i> <sup>[45]</sup> , 2012	Retrospective cohort	120	Cholestasis ( <i>n</i> = 100)	F	EST	109/120 (90.8)	109/120 (90.8)	Bowel perforation ( <i>n</i> = 1, 0.8%) Pancreatitis ( <i>n</i> = 2, 1.7%) Bleeding ( <i>n</i> = 2, 1.7%)
			Biliary pancreatitis ( <i>n</i> = 12)					
			Acute cholangitis ( <i>n</i> = 6)					
			Bile leakage ( <i>n</i> = 2)					
Osoegawa <i>et al</i> <sup>[27]</sup> , 2012	Retrospective cohort	15	N/A	DBE	EST/EPBD±NK	<sup>2</sup> 18/19 (94.7)	<sup>2</sup> 16/18 (88.9)	Bowel perforation ( <i>n</i> = 1, 0.5%)
Sen-Yo <i>et al</i> <sup>[46]</sup> , 2012	Retrospective comparative	65	CBD stone ( <i>n</i> = 38)	AOE	EST/EPBD±NK	60/65 (92.3)	60/60 (100)	Bowel perforation ( <i>n</i> = 1, 1.5%) Pancreatitis ( <i>n</i> = 3, 4.6%) Cholangitis ( <i>n</i> = 2, 3.0%)
			Pancreatico-biliary malignancy ( <i>n</i> = 17)					
			Other malignancy ( <i>n</i> = 2)					
			Chronic pancreatitis ( <i>n</i> = 2)					
			Bile leakage ( <i>n</i> = 2)					
			Others ( <i>n</i> = 4)					
Jang <i>et al</i> <sup>[47]</sup> , 2013	Retrospective cohort	40	CBD stones ( <i>n</i> = 40)	S	EPLBD±NK	40/40 (100)	40/40 (100)	Pancreatitis ( <i>n</i> = 2, 5.0%)
Yao <i>et al</i> <sup>[9]</sup> , 2013	Retrospective cohort	46	CBD stone ( <i>n</i> = 38)	Dual-lumen gastroscop	EST/EPBD	38/46 (82.6)	38/38 (100)	None
			Biliary stricture ( <i>n</i> = 3)					
			Pancreatico-biliary malignancy ( <i>n</i> = 5)					
Kawamura <i>et al</i> <sup>[48]</sup> , 2013	Retrospective comparative	65	CBD stone ( <i>n</i> = 49)	F ( <i>n</i> = 56)	N/A	61/65 (93.8)	51/61 (83.6)	Bowel perforation ( <i>n</i> = 2, 3.1%) Pancreatitis ( <i>n</i> = 4, 6.2%) Bleeding ( <i>n</i> = 1, 1.5%)
			Pancreatico-biliary malignancy ( <i>n</i> = 26)	S ( <i>n</i> = 2) SBE ( <i>n</i> = 3) Others ( <i>n</i> = 4)				
			Benign biliary stricture ( <i>n</i> = 1)					
Kim <i>et al</i> <sup>[49]</sup> , 2014	Retrospective cohort	30	CBD stone ( <i>n</i> = 30)	S	EPLBD±EST	30/30 (100)	30/30 (100)	Pancreatitis ( <i>n</i> = 2, 6.7%) Bleeding ( <i>n</i> = 2, 6.7%)
Iwai <i>et al</i> <sup>[50]</sup> , 2014	Retrospective comparative	19	N/A	SBE	N/A	18/19 (95)	18/18 (100)	None
Cheng <i>et al</i> <sup>[51]</sup> , 2015	Retrospective cohort	77	CBD stone ( <i>n</i> = 77)	DBE	EPLBD/EPBD±NK	73/77 (95)	67/73 (92)	Bowel perforation ( <i>n</i> = 3, 3.8%)

Jang <i>et al</i> <sup>[52]</sup> , 2015	Retrospective cohort	36	CBD stone ( <i>n</i> = 1 <sup>F</sup> 28)  Benign biliary stricture ( <i>n</i> = 6) Pancreaticobiliary malignancy ( <i>n</i> = 1) Post-operative bile leakage ( <i>n</i> = 1)	EPBD± EST	36/36 (100)	32/36 (88.9)	Intestinal mucosal tear ( <i>n</i> = 2, 2.6%) Bowel perforation ( <i>n</i> = 3, 8.3%) Pancreatitis ( <i>n</i> = 2, 5.6%)
Ki <i>et al</i> <sup>[53]</sup> , 2015	Retrospective cohort	72	CBD stone ( <i>n</i> = 1 <sup>F</sup> 55) Cholangitis ( <i>n</i> = 11) CBD stricture ( <i>n</i> = 7) Pancreaticobiliary malignancy ( <i>n</i> = 3) IHD stone ( <i>n</i> = 2)	EST/EPBD	<sup>2</sup> 125/126 (99.2)	<sup>2</sup> 125/125 (100)	Bowel perforation ( <i>n</i> = 1, 0.7%) Pancreatitis ( <i>n</i> = 3, 2.2%) Bleeding ( <i>n</i> = 8, 5.9%)
Nakahara <i>et al</i> <sup>[54]</sup> , 2015	Retrospective cohort	25	CBD stone ( <i>n</i> = 15) Pancreaticobiliary malignancy ( <i>n</i> = 7) Chronic pancreatitis ( <i>n</i> = 3)	AOE EST/EPBD	<sup>2</sup> 26/30 (86.7)	<sup>2</sup> 26/26 (100)	Pancreatitis ( <i>n</i> = 1, 3.3%)
Bove <i>et al</i> <sup>[17]</sup> , 2015	Retrospective cohort	713	CBD stone ( <i>n</i> = 365)  Obstructive jaundice ( <i>n</i> = 177) Acute cholangitis ( <i>n</i> = 61) Chronic pancreatitis ( <i>n</i> = 55) Biliary pancreatitis ( <i>n</i> = 21) Benign biliary stricture ( <i>n</i> = 9) Others ( <i>n</i> = 5)	S ( <i>n</i> = 600) F ( <i>n</i> = 18)  EST	618/713 (86.7)	580/618 (93.8)	Bowel perforation ( <i>n</i> = 22, 3.1%) Pancreatitis ( <i>n</i> = 5, 0.7%)  Bleeding ( <i>n</i> = 11, 1.5%) Mortality ( <i>n</i> = 2, 0.3%)
Wu <i>et al</i> <sup>[18]</sup> , 2016	Retrospective cohort	135	CBD stone/cholangitis Benign biliary stricture	S EST+EPBD	120/135 (88.8)	117/135 (86.3)	<sup>2</sup> Bowel perforation ( <i>n</i> = 1, 0.7%) Pancreatitis ( <i>n</i> = 9, 4.1%) Bleeding ( <i>n</i> = 2, 0.9%)
Park <i>et al</i> <sup>[19]</sup> , 2016	Retrospective cohort	165	CBD stone ( <i>n</i> = 1 <sup>F</sup> 133)  Benign biliary stricture ( <i>n</i> = 21)	EPBD±NK	151/165 (91.5)	144/151 (95.4)	Bowel perforation ( <i>n</i> = 3, 1.8%) Pancreatitis ( <i>n</i> = 13, 7.9%)

			Pancreaticobiliary malignancy ( <i>n</i> = 11)					Hyperamylasemia ( <i>n</i> = 22, 13.3%)
Wang <i>et al</i> <sup>[28]</sup> , 2016	Retrospective cohort	18	CBD stone ( <i>n</i> = 15)	Dual-lumen gastroscope	EST/EPBD	15/18 (83.3)	15/15 (100)	Pancreatitis ( <i>n</i> = 2, 11.1%) Bleeding ( <i>n</i> = 1, 5.6%)
			Pancreaticobiliary malignancy ( <i>n</i> = 3)					
Wang <i>et al</i> <sup>[29]</sup> , 2016	Retrospective cohort	52	CBD stone ( <i>n</i> = 38)	C ( <i>n</i> = 31)	EST/EPBD±NK	50/52 (96.2)	50/52 (96.2)	Pancreatitis ( <i>n</i> = 2, 3.8%) Hyperamylasemia ( <i>n</i> = 2, 3.8%)
			Biliary stricture ( <i>n</i> = 9)	F ( <i>n</i> = 13)				
			Pancreaticobiliary malignancy ( <i>n</i> = 5)	S ( <i>n</i> = 11)				
Shimatani <i>et al</i> <sup>[25]</sup> , 2016	Prospective cohort	26	Cholangitis ( <i>n</i> = 13)	DBE	EST	25/26 (96.2)	25/25 (100)	Bowel perforation ( <i>n</i> = 2, 7.7%) Pancreatitis ( <i>n</i> = 5, 19.2%) Cholangitis ( <i>n</i> = 1, 3.8%) Aspiration pneumonia ( <i>n</i> = 1, 3.8%)
			Hepatobiliary disorder ( <i>n</i> = 4)					
			Obstructive jaundice ( <i>n</i> = 4)					
			CBD stone ( <i>n</i> = 2)					
			Others ( <i>n</i> = 3)					
Shimatani <i>et al</i> <sup>[55]</sup> , 2017	Retrospective cohort	11	CBD stone ( <i>n</i> = 7)	DBE	EST	11/11 (100)	11/11 (100)	None
			Obstructive jaundice ( <i>n</i> = 2)					
			Others ( <i>n</i> = 2)					
Yane <i>et al</i> <sup>[26]</sup> , 2017	Retrospective cohort	20	CBD stone	SBE	N/A	20/20 (100)	19/20 (95)	Bowel perforation ( <i>n</i> = 2, 1.0%) Pancreatitis ( <i>n</i> = 3, 1.5%) Cholangitis ( <i>n</i> = 4, 2.0%) Cholecystitis ( <i>n</i> = 1, 0.5%)
			Bile duct stricture					
			Anastomosis site stricture					
Li <i>et al</i> <sup>[56]</sup> , 2017	Retrospective cohort	49	CBD stone ( <i>n</i> = 49)	S	EPBD	N/A	42/49 (85.7)	Pancreatitis ( <i>n</i> = 3, 6.1%)
Han <i>et al</i> <sup>[57]</sup> , 2018	Retrospective cohort	15	CBD stone ( <i>n</i> = 15)	<sup>1</sup> F	EST/EPBD±NK	15/15 (100)	15/15 (100)	Pancreatitis ( <i>n</i> = 1, 6.7%)

<sup>1</sup>Cap-fitted forward-viewing endoscope;

<sup>2</sup>The data are per procedure, not per patient. A-loop: Afferent loop; ERCP: Endoscopic retrograde cholangiopancreatography; N/A: Not available; RUQ: Right upper quadrant; EST: Endoscopic sphincterotomy; NK: Needle knife; EPBD: Endoscopic papillary balloon dilation; EPLBD: Endoscopic papillary large balloon dilation; CBD: Common bile duct; IHD: Intrahepatic duct; F: Forward-viewing endoscope; S: Side-viewing endoscope; C: Colonoscope; SBE: Single-balloon enteroscope; DBE: Double-balloon enteroscope; AOE: Anterior oblique-viewing endoscope.

As an introduction to balloon-assisted endoscopy, the double-balloon enteroscope or single-balloon enteroscope have been increasingly used to perform ERCP in surgically altered anatomy, including Billroth II gastrectomy<sup>[5,6]</sup>. The success rates of ERCP in Billroth II gastrectomy cases by balloon-assisted enteroscopy have been reported from 95.0% to 100%, and seem to be comparable with those of conventional side-viewing endoscopy or forward-viewing endoscopy<sup>[25,26]</sup>. Balloon-assisted enteroscope has significant benefit to overcome the sharp curve of the anastomosis site and advance much deeper into the small intestine than conventional side-viewing endoscope or forward-viewing endoscope<sup>[5,27]</sup>. However, ERCP by balloon-assisted enteroscopy is technically demanding and requires expertise and specialized equipments. Balloon-assisted enteroscope is also forward-viewing instrument, which has disadvantages of difficulty in obtaining an en face view of the papilla. Therefore, a head to head comparison of outcomes between different types of endoscopy with a randomized controlled trial (RCT) is needed in the future.



**Table 2** Detailed characteristics of the most recently published studies with more than 100 patients

	<b>Bove <i>et al</i><sup>[17]</sup>, 2015</b>	<b>Wu <i>et al</i><sup>[18]</sup>, 2016</b>	<b>Park <i>et al</i><sup>[19]</sup>, 2016</b>
	<b>(n = 713)</b>	<b>(n = 135)</b>	<b>(n = 165)</b>
Study design	Retrospective cohort in single center	Retrospective cohort in single center	Retrospective cohort in 5 centers
Male gender, n (%)	567 (79.5)	N/A	116 (70.3)
Age (yr), n (%) or mean $\pm$ SD	> 60 yr, 565 (79.2)	N/A	71.1 $\pm$ 10.0
Type of endoscope	Side-viewing or forward-viewing	Side-viewing	Cap-fitting forward-viewing
Type of sphincter therapy	EST	EST	EPBD $\pm$ NK
Success of afferent loop intubation, n (%)	618/713 (86.7)	120/135 (88.8)	151/165 (91.5)
Success of selective cannulation, n (%)	580/618 (93.8)	117/120 (97.5)	144/151 (95.4)
Bowel perforation, n (%)	22/713 (3.1)	1/135 (0.7)	3/165 (1.8)
Post-ERCP pancreatitis, n (%)	5/713 (0.7)	N/A	13/165 (7.9)
Bleeding, n (%)	11/713 (1.5)	N/A	-
Mortality, n (%)	2/713 (0.3)	-	-

ERCP: Endoscopic retrograde cholangiopancreatography; SD: Standard deviation; EST: Endoscopic sphincterotomy; NK: Needle knife; EPBD: Endoscopic papillary balloon dilation; N/A: Not available.

Recently, the advent of new types of endoscopes, such as a dual lumen or multiple bending endoscope, has allowed successful afferent loop intubation and selective cannulation<sup>[10,28]</sup>. The use of dual lumen endoscope has potential advantage that the cooperation of two instruments through different channels can facilitate papillary cannulation in cases with difficult anatomy such as periampullary diverticulum and surgical altered anatomy. Unfortunately, the success rate of these procedures is not significantly higher than that of conventional side-viewing endoscopy, easily available forward-viewing endoscopy or standard colonoscopy (dual lumen endoscope, 82.8%; multiple bending endoscope, 92.9% *vs* conventional side-viewing endoscopy, 93.8%-97.5%; forward-viewing endoscopy, 95.4%; standard colonoscopy, 96.2%)<sup>[9,10,17-19,28,29]</sup>. Until now, there has been no large-scale retrospective cohort study or prospective comparative study. Therefore, the new types of endoscopes are practically and economically limited for widespread use.

Another issue, the choice of sphincter therapy, has also been a matter of debate regarding ERCP with Billroth II gastrectomy patients. Traditionally, the performance of sphincterotomy in Billroth II cases is difficult due to its reverse position of the biliary and pancreatic duct. The difficulty of sphincterotomy in optimal direction is associated with the risk of bowel perforation or bleeding. So, there have been continued considerable efforts to perform effective and safe sphincter therapy. Dedicated sphincterotomes for Billroth II anatomy such as inverted sphincterotome or S-shaped sphincterotome have been developed and widely used. Recently, the use of EPBD for sphincter management in Billroth II patients has been on the increase. EPBD is particularly useful in ERCP with a forward-viewing endoscope since sphincterotomy may be difficult with this scope which does not have an elevator. Furthermore, in cases with large CBD stones, application of EPLBD can help to efficiently remove these stones in Billroth II gastrectomy cases<sup>[12,30]</sup>. Therefore, the increasing use of balloon dilator has been the general trend in sphincter therapy in recent years.

This study has potential limitations that should be discussed. First, in this study, it is not sufficiently and clearly described a recent trend toward a better outcome with novel technologies in ERCP in Billroth II gastrectomy patients. Because most of studies regarding novel technologies were case report, case series, and animal study, they were excluded from current systematic review. This point is major limitation of current study. Second, the studies included in the current systematic review were retrospective, observatory publications from more than 30 years with heterogeneous indications for ERCP. The performance bias of ERCP according to the endoscopist's experience and technique and operative consideration, including the duration and type of Billroth II operation (antecolic or retrocolic, anisoperistaltic or isoperistaltic), were not described. The older studies can lead to bias because there are the difference of the technological advance such as endoscopic instruments and skill, overall knowledge and experience of endoscopists in performing ERCP of Billroth II gastrectomy patients between the past and the present.

In summary, conventional side-viewing endoscopy remains the most commonly used type of endoscopy for ERCP in Billroth II gastrectomy cases. Forward-viewing

**Table 3 Results of the systematic review**

	No. (%)
Study design, <i>n</i> (%)	
Retrospective cohort	36/43 (83.7)
Retrospective comparative	4/43 (9.3)
Prospective comparative	2/43 (4.7)
Prospective cohort	1/43 (2.3)
Total number of identified patients	2669
<sup>1</sup> Type of endoscope, <i>n</i> (%)	
Side-viewing endoscope	1432/2575 (55.6)
Forward-viewing endoscope	664/2575 (25.8)
Balloon-assisted enteroscope	197/2575 (7.7)
Anterior oblique-viewing endoscope	169/2575 (6.6)
Dual-lumen endoscope	64/2575 (2.5)
Colonoscope	31/2575 (1.2)
Multiple bending endoscope	14/2575 (0.5)
Others	4/2575 (0.2)
Overall success of afferent loop intubation, <i>n</i> (%)	2437/2669 (91.3)
Overall success of selective cannulation, <i>n</i> (%)	2346/2437 (87.9)
Overall adverse events, <i>n</i> (%)	195 (7.3)
Bowel perforation	74 (2.8)
Post-ERCP pancreatitis	65 (2.4)
Bleeding	37 (1.4)
Mortality	9 (0.3)
<sup>2</sup> Others	10 (0.4)

<sup>1</sup>Total number can be changed due to unavailable or incomplete specific data;

<sup>2</sup>Cholangitis (*n* = 7), respiratory insufficiency (*n* = 1), aspiration pneumonia (*n* = 1), and cholecystitis (*n* = 1). ERCP: Endoscopic retrograde cholangiopancreatography.

endoscopy has been increasingly used to perform ERCP in Billroth II gastrectomy cases because of its advantages, including easy availability and good visual field, as well as the additional advantage of the transparent cap being fitted to the distal end of the endoscope. In recent years, new types of endoscopy, including balloon-assisted enteroscopy, anterior oblique-viewing endoscopy, dual-lumen endoscopy, and multiple bending endoscopy, have been introduced and performed with ERCP safely and effectively. There have also been various types of sphincter therapy applied, including EST, EPBD, and EPLBD, with or without precutting by NK, and the use of diverse types of accessories. As considerable efforts of worldwide investigators have been applied for safe and effective ERCP in this population, the success rate of the procedure and occurrence of adverse events have been improving. In addition, a RCT is required to evaluate the optimal type of endoscopy and sphincter therapy for ERCP in Billroth II gastrectomy patients in the future.

**Table 4** Subgroup analysis according to the type of endoscope

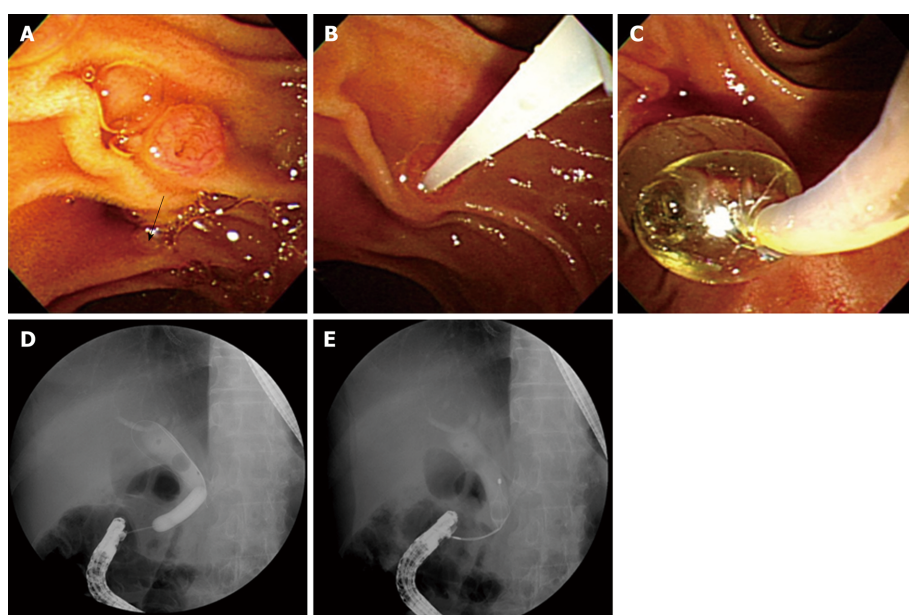
	Side-viewing endoscope	Forward-viewing endoscope	Balloon-assisted enteroscope	Oblique-viewing endoscope	Dual-lumen endoscope
	( <i>n</i> = 1432)	( <i>n</i> = 664)	( <i>n</i> = 197)	( <i>n</i> = 169)	( <i>n</i> = 64)
Afferent loop intubation, <i>n</i> (%)	1406 (98.2)	647 (97.4)	188 (95.4)	159 (94.1)	53(82.8)
Selective cannulation, <i>n</i> (%)	1340 (95.3)	616 (95.2)	179 (97.5)	155 (97.5)	53 (100)
Adverse events, <i>n</i> (%)	113 (7.9)	47 (7.1)	14 (7.1)	6 (3.6)	3 (4.7)
Bowel perforation, <i>n</i> (%)	51 (3.6)	11 (1.7)	8 (4.1)	2 (1.2)	2 (3.1)
Post-ERCP pancreatitis, <i>n</i> (%)	26 (1.8)	27 (4.1)	6 (3.0)	3 (1.8)	1 (1.6)
Bleeding, <i>n</i> (%)	27(1.9)	9 (1.4)	-	1 (0.6)	-
Mortality, <i>n</i> (%)	9 (0.6)	-	-	-	-

ERCP: Endoscopic retrograde cholangiopancreatography.

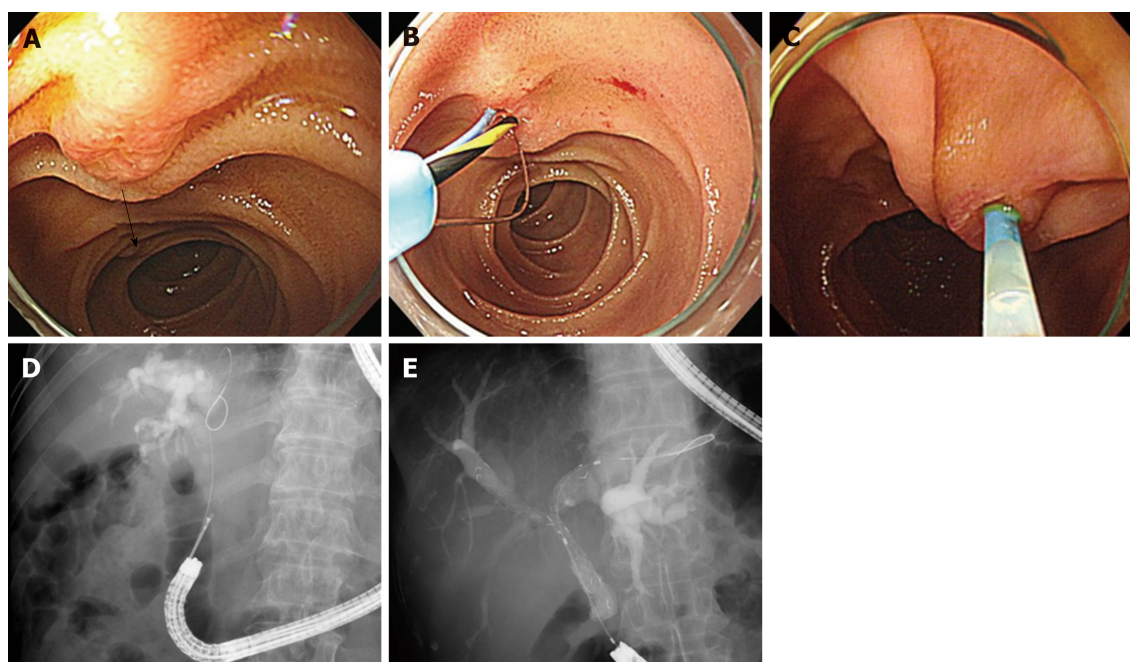
**Table 5** Subgroup analysis according to the sphincter management methods

	EST	EST+EPBD	EPBD	EPLBD
	( <i>n</i> = 1478)	( <i>n</i> = 598)	( <i>n</i> = 246)	( <i>n</i> = 171)
<sup>1</sup> Clinical success, <i>n</i> (%)	1268 (85.8)	546 (91.3)	214 (87.0)	160 (93.6)
Adverse events, <i>n</i> (%)	103 (7.0)	38 (6.4)	21 (8.5)	10 (5.8)
Bowel perforation, <i>n</i> (%)	51 (3.5)	8 (1.3)	5 (2.0)	3 (1.8)
Post-ERCP pancreatitis, <i>n</i> (%)	18 (1.2)	22 (3.7)	16 (6.5)	4 (2.3)
Bleeding, <i>n</i> (%)	25 (1.7)	8 (1.3)	-	3 (1.8)
Mortality, <i>n</i> (%)	9 (0.6)	-	-	-

<sup>1</sup>Clinical success was defined as the achievement of the planned therapeutic goals including bile duct stone clearance, endobiliary biopsy, biliary stent or nasobiliary catheter insertion. The number of patients is much decreased because three studies, unavailable sphincter management information, were excluded from the subgroup analysis. ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilation; EPLBD: Endoscopic papillary large balloon dilation.



**Figure 2** Side-viewing endoscopy. A: Naïve papilla; *En face* view can be obtained with ease. The direction of bile duct is reversed (arrow); B: Selective cannulation can be achieved with assistance of elevator; C: Sphincter management with papillary balloon dilation; endoscopic view; D: Sphincter management with papillary balloon dilation; fluoroscopic view; E: Common bile duct stone was removed by basket.



**Figure 3** Cap-fitting forward-viewing endoscopy. A: Naïve papilla; It is difficult to obtain *en face* view. The direction of bile duct is reversed (arrow); B: Gastroscop at 7 o'clock position working channel; Sphincter management with inverted sphincterotome; C: Pediatric colonoscope at 5 o'clock position working channel; D: Endobiliary biopsy was performed in distal common bile duct stricture; E: Bilateral uncovered metal stents were inserted in the malignant hilar stricture.

## ARTICLE HIGHLIGHTS

### Research background

Endoscopic retrograde cholangiopancreatography (ERCP) in patients who have a Billroth II gastrectomy has been considered a difficult procedure due to the surgically altered anatomy. The difficulties of ERCP in patients with Billroth II gastrectomy include the intubation of the afferent loop, visualization of the papilla, selective cannulation of the bile duct, and optimal sphincter management due to the reverse direction of the papilla. To perform safe and effective ERCP in Billroth II gastrectomy cases, considerable efforts have been put in several ways including the choice of endoscope and sphincter management. However, there has been a paucity of comparative studies on the efficacy and safety regarding ERCP in Billroth II gastrectomy.

### Research motivation

At present, comparative studies on the efficacy and safety of ERCP in Billroth II gastrectomy cases are lacking because of practical and ethical limitations due to procedure-related morbidity and mortality. This systematic and comprehensive review was performed to obtain a recent perspective on ERCP in Billroth II gastrectomy patients.

### Research objectives

The main objective of the study was to assess the efficacy and safety of ERCP in Billroth II gastrectomy patients. In detail, the assessment of success rate of afferent loop intubation and selective cannulation, and rate of adverse events including bowel perforation, post-ERCP pancreatitis, bleeding, cardiopulmonary events, and mortality was performed. In addition, the assessment of these outcomes according to each type of endoscopy and sphincter management methods was performed.

### Research methods

A systematic review was performed on the literatures that evaluated the outcomes of ERCP in Billroth II gastrectomy patients. Electronic databases were searched, including PubMed, EMBASE, and Cochrane Library. The outcomes of afferent loop intubation and selective cannulation, and occurrence of adverse events were assessed.

### Research results

A total of 43 studies involving 2669 patients were included. The overall success rate of afferent loop intubation was 91.3% (2437/2669), and the overall success rate of selective cannulation was 87.9% (2346/2437). A total of 195 cases (7.3%) of adverse events occurred. Bowel perforations occurred in 74 cases (2.8%), post-ERCP pancreatitis in 65 cases (2.4%), bleeding in 37 cases (1.4%), mortality in 9 cases (0.3%).

### Research conclusions

This systematic review showed that the performance of ERCP in the Billroth II gastrectomy



patients has been improving with choice of endoscope and sphincter management. To determine the optimal method to perform safe and effective ERCP in Billroth II gastrectomy patients, more comparative studies are needed in the future.

### Research perspectives

The success of ERCP in Billroth II gastrectomy has been improving with technical advance. Future research is needed to explore the optimal approach in performance of ERCP in Billroth II gastrectomy cases.

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